REMARKS

Claims 32-50 were pending in the application. Claims 33-39 were withdrawn from consideration as being directed to non-elected subject matter. Claims 32, 43, and 47 have been amended. Support for the amendments to claims 32, 43, and 47 can be found throughout the specification, including at least at line 16, lines 16-17 and page 18, lines 16-17. New claims 51-54 have been added. Support for the new claims can be found in the claims as originally filed and throughout the specification. Additional support for new claim 51 can be at least at page 16, lines 29-37 of the specification. Thus, upon entry of this Amendment, claims 32-54 are pending in the application.

No new matter has been added. Applicants request that the amendments to the specification and claims be entered. The foregoing claim amendments and cancellation should in no way be construed as an acquiescence to any of the Examiner's rejections and were made solely to expedite prosecution of the present application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Restriction Requirement

Applicant gratefully acknowledges the Examiner's rejoining of Groups I-IV issued in the corresponding Restriction Requirement, as indicated at page 3 of the Office Action mailed July 15, 2003.

Rejection of Claims 32, 40-50 Under 35 U.S.C. 112, First Paragraph

Claims 32 and 40-50 are rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the enablement requirement. The Examiner states that the instant specification "is not sufficient to overcome the art recognized unpredictable nature of transgenic animal phenotype." The Examiner also asserts that, "one skilled in the art

would have to engage in an undue amount of experimentation to use the claimed invention at the time of filing." Applicants respectfully traverse this rejection.

As amended, the claimed invention describes a non-human transgenic animal comprising a transgene comprising a nucleic acid molecule encoding a fusion protein which activates transcription of a gene of interest operatively linked to a target DNA sequence to which the fusion protein binds, the fusion protein comprising a first polypeptide comprising a DNA binding domain operatively linked to a second polypeptide comprising a transcriptional activation domain, wherein the transcriptional activation domain comprises at least one copy of a mutated acidic region of herpes simplex virus virion protein 16 (HSV VP16), the mutated acidic region consisting of amino acid positions 436 to 447 of HSV VP16 (SEQ ID NO: 1) and having an amino acid substitution at position 442 as compared to wild type HSV VP16, the transgene being in a form suitable for expression of the fusion protein in cells of the non-human transgenic animal. In one embodiment, the transcriptional activation domain consists of three copies of VP16. In another embodiment, the transcriptional activation domain consists of four copies of VP16. In one embodiment of the invention, the first polypeptide is a Tet repressor. In another embodiment, the first polypeptide is a mutated Tet repressor that binds to *tet*O sequences in the presence, but not in the absence, of tetracycline or a tetracycline analogue. In some embodiments, the invention includes first polypeptides which consist of GAL4, LexA, LacR, or steroid hormone receptors.

The specification provides detailed guidance regarding how to make nucleic acid molecules encoding the transcriptional activator fusion proteins of the invention, including providing specific examples (see *e.g.*, pages 10, line 36 to page 11, line 19 and Example 1 at pages 33-34). The specification further provides guidance regarding how to incorporate such nucleic acid molecules into vectors to be used for expression of the fusion protein of the invention in a cell, wherein the cell is used for making transgenic or

homologous recombinant organisms (see *e.g.*, page 11, line 23 to page 12, line 34 and page 13, line 19 to page 15, line 26).

The instant specification teaches how to prepare the claimed transgenic and homologous recombinant organisms (see *e.g.*, page 15, line 29 through page 17, line 31). For example, the specification teaches that a transgenic animal of the invention can be created by introducing the claimed fusion protein into the male pronuclei of a fertilized oocyte, and allowing the oocyte to develop in a pseudopregnant mother (Page. 16, lines 4-8). At page 16, lines 9-13, Applicant cites references which describes methods of creating transgenic animals which are standard in the art, including how to make transgenic mice. Applicant also teaches that founder animals can be used to identify animals which carry the transgene, and that these founder animals can be used to breed a line of animals (page 16, lines 13-17).

The Examiner is of the opinion that at the time of filing the application, the state of the art regarding transgenic animals was unpredictable, and cites a number of references in support of this assertion. The Examiner cites Mullins as teaching difficulties in producing transgenic large mammals, in comparison to mice. The Examiner points to the teaching in Mullins which describes the failure of a construct designed for ovine to work in mice. Applicants submit that in contrast to the Examiner's assertion, the teachings of Mullins is an example of <u>successful</u> expression of a transgene in sheep, demonstrating that, at the time of the invention, technology for successfully making transgenic sheep was available in the art. The fact that Mullins observed lower expression in transgenic mice when the identical sheep construct was used in the mice simply indicates that a transgene designed for one species may not be optimal in another species. In conclusion of these findings, Mullins qualifies the predictive value of mouse models in studying transgene constructs for other species, stating, "the generation of transgenic mice may, in certain cases, only be a guide to the potential success of a

transgene construct in another species" (page S38, second column, first paragraph, last sentence). Applicant submits that one of ordinary skill in the art would recognize that the transgene construct to be used to make a transgenic animal of the invention would depend on the type of animal. Furthermore, the specification teaches that the design of expression vectors depends on the particular host cell in which the protein is to be expressed (see page 11, lines 34-36) and various promoters for expression in different species of host cells were known in the art.

The Examiner cites Seidel as teaching challenges associated with transgenic livestock species, including the length of time which it takes to produce and characterize such transgenic animals. These teachings of Seidel at most point out that creating transgenic animals in species other than mice may be more time-consuming or problematic from a logistic standpoint, but the fact that more time may be necessary to make a non-murine transgenic animal or that it may be logistically more difficult does not mean that such animals are not enabled by the invention. In fact, Seidel teach that in five separate studies utilizing cattle, goats, sheep and swine, between 3.6 and 9.8% of the offspring attained contained the transgene, and between 37.5 and 55.6% of these transgenic offspring expressed the transgene (Page 28, Table 2). Thus, the reference cited by the Examiner actually demonstrates that technologies for successfully making transgenic animals in non-mouse species were available in the art at the time of the invention. It would therefore not have required undue experimentation on the part of one skilled in the art to obtain a transgenic animal as claimed in the invention.

Finally, the Examiner cites Hammer *et al.* in support of the "unpredictability" in making transgenic animals. The Examiner relies on Hammer et al. for teaching that "integration of a transgene into alternative species may result in widely different phenotypic responses." Applicant submits that Hammer *et al.*, which was published in 1990, is not representative of the predictability of transgenic animals at the time of filing.

In response to the Examiner's assertion that the Hammer reference shows that HLA-B27 mice did not show any disease phenotypes while the HLA-B27 rats did demonstrate associated diseases, Applicant points out that Hammer specifically teaches not only that the expression and activity of the B27 transgene in mice was "physiologically normal", but further suggest that the arthritis phenotype that the researchers were expecting in the mice upon expression of the transgene *may not be related to B27 expression at all.* Hammer teaches that several arthritic diseases readily inducible in rats cannot be induced in mice, *regardless of transgene expression* (page 1099, column 2, paragraph 2). This reference neither teaches nor suggests that the phenotypic difference between rats and mice was due to differential expression or activity of the B27 transgene in these animals due to the unpredictability of transgene expression and integration. In fact, this reference is an example of successful expression of a transgene in two different species, rats and mice.

At the time of filing the priority application, the teachings of the specification with regard to the construction of transgenic organisms (see *e.g.*, page 16), had been utilized by many groups in the production of a variety of transgenic organisms, including mice, rats, pigs, sheep, and cows. A PubMed database search of the terms "transgenic AND mice" limited to the year 1997 (the year of filing of the priority document) resulted in 2,220 hits, supporting Applicant's assertion that methods for producing transgenic animals including mice were known at the time of filing. Applicants also refer the Examiner to examples of non-murine transgenic animals which were described in the literature at the time of filing the priority application:

A and B. WO 92/22646 and WO 93/25017, entitled "Production of Human Hemoglobin Transgenic Pigs" (attached herewith as Appendix A and B, respectively) teach the production of transgenic pigs expressing the human hemoglobin gene.

C. U.S. patent no. 5,366,894, entitled "Protein Production" (attached herewith as Appendix C) teaches a method of producing transgenic sheep who express a desired transgene in their milk, wherein the protein can be easily collected and purified.

D. Fodor *et al.* (1994) *PNAS* 91:11153-11157 (attached herewith as Appendix D) who describe the production of transgenic pigs which express human CD59;

E. Kroshus *et al.* (1996) *Transplantation* 61:1513-1521 (attached herewith as Appendix E) who describe transgenic pigs who express human CD59 on their organs in order to decrease the chance of rejection in xenotransplantation. The Kroshus paper follows the experiment described in the above-mentioned Fodor publication, and demonstrates that the transgenic pigs originally described in Fodor were successfully used in subsequent studies;

F. Wall *et al.* (1996) *Transgenic Research* 5:67-72 (attached herewith as Appendix F) who describe transgenic sheep which express the whey acidic protein (WAP); and

G. PCT publication no. WO 97/19589 (attached herewith as Appendix G) teaches methods of producing transgenic goats, including how to obtain goat stem cells.

Each of these references demonstrates the efficacy and predictability of the methodologies taught in the instant specification and known at the time of filing in the production of transgenic animals. These references also demonstrate that one of ordinary skill in the art would not have to perform undue experimentation to use the claimed invention. It is therefore clear from the teachings of these references that the methodologies taught by Applicants are valid for the production of animals transgenic for the transactivator fusion protein of the invention.

As discussed in the specification, standard molecular biology techniques are used to construct the DNA and vectors of the invention. Furthermore, as discussed in the specification, standard techniques known in the art are used to prepare the transgenic organisms of the invention. It is well established that a patent specification need not

teach, and preferably omits, that which is well known in the art. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81 (Fed. Cir. 1986). In view of the teachings in the specification and the general knowledge in the art regarding how to make and use transgenic organisms, the specification has provided sufficient guidance to the ordinarily skilled artisan as to how to make and use the invention. Accordingly, the specification meets the enablement requirement.

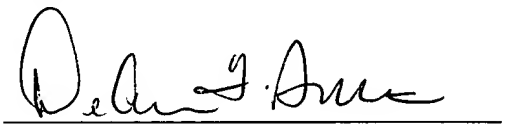
In view of the above arguments, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection of claims under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 32, 40-50 Under 35 U.S.C. 112, Second Paragraph

Claims 32 and 40-50 are rejected under 35 U.S.C. § 112, first paragraph as being indefinite to particularly point out the claimed invention. Specifically, the Examiner states use of the phrase "a fusion protein which activates transcription" makes the claims indefinite "because it is unclear the transcription of which gene is activated by the fusion protein." Claims 32, 43, and 47 have been amended to specify that the fusion protein of the invention activates transcription of a gene of interest operatively linked to a target DNA sequence to which a transactivator fusion protein binds. Applicants submit that amended claims 32, 43, and 47 completely define which gene is being activated by the fusion protein. In view of the amendment and the clear and definite description of genes which can be activated using the transactivator protein provided in the specification, Applicants respectfully request that the Examiner withdraw this 112 rejection.

If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicants' Attorney at (617) 227-7400.

Respectfully submitted,



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Appendix A

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(54) Title: PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS

(57) Abstract

The present invention relates to the use of transgenic pigs for the production of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.

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PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS

1. INTRODUCTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.

2. BACKGROUND OF THE INVENTION

2.1. HEMOGLOBIN

Oxygen absorbed through the lungs is carried by hemoglobin in red blood cells for delivery to tissues throughout the body. At high oxygen tensions, such as those found in the proximity of the lungs, oxygen binds to hemoglobin, but is released in areas of low oxygen tension, where it is needed.

Each hemoglobin molecule consists of two alpha globin and two beta globin subunits. Each subunit, in turn, is noncovalently associated with an iron-containing heme group capable of carrying an oxygen molecule. Thus, each hemoglobin tetramer is capable of binding four molecules of oxygen. The subunits work together in switching between two conformational states to facilitate uptake and release of oxygen at the lungs and tissues, respectively. This effect is commonly referred to as heme-heme interaction or cooperativity.

The hemoglobins of many animals are able to interact with biologic effector molecules that can further enhance oxygen binding and release. This enhancement is manifested in changes which affect the allosteric equilibrium between the two conformational

states of hemoglobin. For example, human and pig hemoglobin can bind 2, 3 diphosphoglycerate (2,3 DPG), which influences the equilibrium between the two conformational states of the tetramer and has the net effect of lowering the overall affinity for oxygen at the tissue level. As a result, 2,3-DPG increases the efficiency of oxygen delivery to the tissues.

2.2. GLOBIN GENE EXPRESSION

Hemoglobin protein is expressed in a tissue specific manner in red blood cells where it accounts for approximately ninety percent of total cellular protein. Thus, red blood cells, which have lost their nucleus and all but a minimal number of organelles, are effectively membrane-enclosed packets of hemoglobin dedicated to oxygen transfer.

Humans and various other species produce different types of hemoglobin during embryonic, fetal, and adult developmental periods. Therefore, the factors that influence globin gene expression must be able to achieve tissue specific control, quantitative control, and developmentally regulated control of globin expression.

Human globin genes are found in clusters on chromosome 16 for alpha (α) globin and chromosome 11 for beta (β) globin. The human beta globin gene cluster consists of about 50 kb of DNA that includes one embryonic gene encoding epsilon (ϵ) globin, two fetal genes encoding gamma (γ) G and gamma A globin, and two adult genes encoding delta (δ) and beta (β) globin, in that order (Fritsch et al., 1980, Cell 19:959-972).

It has been found that DNA sequences both upstream and downstream of the β globin translation initiation site are involved in the regulation of β

globin gene expression (Wright et al., 1984, Cell 38:263). In particular, a series of four Dnase I super hypersensitive sites (now referred to as the locus control region, or LCR) located about 50 kilobases upstream of the human beta globin gene are extremely important in eliciting properly regulated beta globin-locus expression (Tuan et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 83:1359-1363; PCT Patent Application WO 8901517 by Grosveld; Behringer et al., 1989, Science 245:971-973; Enver et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037; Hanscombe et al., 1989, Genes Dev. 3:1572-1581; Van Assendelft et al., 1989, Cell 56:967-977; Grosveld et al., 1987, Cell 51:975-985).

2.3. THE NEED FOR A BLOOD SUBSTITUTE

Recently, the molecular aspects of globin gene expression have met with even greater interest as researchers have attempted to use genetic engineering to produce a synthetic blood that would avoid the pitfalls of donor generated blood. In 1988, between 12 million and 14 million units of blood were used in the United States alone (Andrews, February 18, 1990, New York Times), an enormous volume precariously dependent on volunteer blood donations. About 5 percent of donated blood is infected by hepatitis virus (Id.) and, although screening procedures for HIV infection are generally effective, the prospect of contracting transfusion related A.I.D.S. remains a much feared possibility. Furthermore, transfused blood must be compatible with the blood type of the transfusion recipient; the donated blood supply may be unable to provide transfusions to individuals with rare blood types. In contrast, hemoglobin produced by genetic engineering would not require blood type

matching, would be virus-free, and would be available in potentially unlimited amounts. Several research groups have explored the possibility of expressing hemoglobin in microorganisms. For example, see International Application No. PCT/US88/01534 by Hoffman and Nagai, which presents, in working examples, production of human globin protein in E. coli.

2.4. TRANSGENIC ANIMALS

A transgenic animal is a non-human animal containing at least one foreign gene, called a transgene, in its genetic material. Preferably, the transgene is contained in the animal's germ line such that it can be transmitted to the animal's offspring. A number of techniques may be used to introduce the transgene into an animal's genetic material, including, but not limited to, microinjection of the transgene into pronuclei of fertilized eggs and manipulation of embryonic stem cells (U.S. Patent No. 4,873,191 by Wagner and Hoppe; Palmiter and Brinster, 1986, Ann. Rev. Genet. 20:465-499; French Patent Application 2593827 published August 7, 1987). Transgenic animals may carry the transgene in all their cells or may be genetically mosaic.

Although the majority of studies have involved transgenic mice, other species of transgenic animal have also been produced, such as rabbits, sheep, pigs (Hammer et al., 1985, Nature 315:680-683) and chickens (Salter et al., 1987, Virology 157:236-240). Transgenic animals are currently being developed to serve as bioreactors for the production of useful pharmaceutical compounds (Van Brunt, 1988, Bio/Technology 6:1149-1154; Wilmut et al., 1988, New Scientist (July 7 issue) pp. 56-59).

Methods of expressing recombinant protein via transgenic livestock have an important theoretical advantage over protein production in recombinant bacteria and yeast; namely, the ability to produce large, complex proteins in which post-translational modifications, including glycosylation, phosphorylation, subunit assembly, etc. are critical for the activity of the molecule.

In practice, however, the creation of transgenic livestock has proved problematic. Not only is it technically difficult to produce transgenic embryos, but mature transgenic animals that produce significant quantities of recombinant protein may prove inviable. In pigs in particular, the experience has been that pigs carrying a growth hormone encoding transgene (the only transgene introduced into pigs prior to the present invention) suffered from a number of health problems, including severe arthritis, lack of coordination in their rear legs, susceptibility to stress, anoestrus in gilts and lack of libido in boars (Wilmut et al., supra). This is in contrast to transgenic mice carrying a growth hormone transgene, which appeared to be healthy (Palmiter et al., 1982, Nature 300:611-615). Thus, prior to the present invention, healthy transgenic pigs (which efficiently express their transgene(s)) had not been produced.

2.5. EXPRESSION OF GLOBIN GENES IN TRANSGENIC ANIMALS

Transgenic mice carrying human globin transgenes have been used in studying the molecular biology of globin gene expression. A hybrid mouse/human adult beta globin gene was described by Magram et al. in 1985 (Nature 315:338-340). Kollias et al. then reported regulated expression of human gamma-A, beta, and hybrid beta/gamma globin genes in

transgenic mice (1986, Cell 46:89-94). Transgenic mice expressing human fetal gamma globin were studied by Enver et al. (1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037) and Constantoulakis et al. (1991, Blood 77:1326-1333). Autonomous developmental control of human embryonic globin gene switching in transgenic mice was observed by Raich et al. (1990, Science 250:1147-1149).

Transgenic mouse models for a variety of disorders of hemoglobin or hemoglobin expression have been developed, including sickle cell disease (Rubin et al., 1988, Am. J. Human Genet. 42:585-591; Greaves et al., 1990, Nature 343:183-185; Ryan et al., 1990, Science 247:566-568; Rubin et al., 1991, J. Clin. Invest. 87:639-647); thalassemia (Anderson et al., 1985, Ann. New York Acad. Sci. (USA) 445:445-451; Sorenson et al., 1990, Blood 75:1333-1336); and hereditary persistence of fetal hemoglobin (Tanaka et al., 1990, Ann. New York Acad. Sci. (USA) 612:167-178).

Concurrent expression of human alpha and beta globin has led to the production of human hemoglobin in transgenic mice (Behringer et al., 1989, Science 245:971-973; Townes et al., 1989, Prog. Clin. Biol. Res. 316A:47-61; Hanscombe et al., 1989, Genes Dev. 3:1572-1581). It was observed by Hanscombe et al. (supra) that transgenic fetuses with high copy numbers of a transgene encoding alpha but not beta globin exhibited severe anemia and died prior to birth. Using a construct with both human alpha and beta globin genes under the control of the beta globin LCR, live mice with low copy numbers were obtained (Id.). Metabolic labeling experiments showed balanced mouse globin synthesis, but imbalanced human globin

synthesis, with an alpha/beta biosynthetic ratio of about 0.6 (Id.).

3. SUMMARY OF THE INVENTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin and/or human globin. It is based, at least in part, on the discovery that transgenic pigs may be generated that express human hemoglobin in their erythrocytes and are healthy, suffering no deleterious effects as a result of heterologous hemoglobin production.

In particular embodiments, the present invention provides for transgenic pigs that express human globin genes. Such animals may be used as a particularly efficient and economical source of human hemoglobin, in light of (i) the relatively short periods of gestation and sexual maturation in pigs; (ii) the size and frequency of litters, (iii) the relatively large size of the pig which provides proportionately large yields of hemoglobin; and (iv) functional similarities between pig and human hemoglobins in the regulation of oxygen binding affinity which enables the transgenic pigs to remain healthy in the presence of high levels of human hemoglobin.

The present invention also provides for recombinant nucleic acid constructs that may be used to generate transgenic pigs. In preferred embodiments, such constructs place the human alpha and beta globin genes under the same promoter so as to avoid deleterious effects of globin chain imbalance and/or titration of transcription factors due to constitutive β-globin promoter activity in an inappropriate cell type (e.g. a primitive erythrocyte).

In an additional embodiment, the present invention provides for a hybrid hemoglobin that comprises human α globin and pig β globin. The whole blood from transgenic pigs expressing this hybrid hemoglobin appears to exhibit a P_{50} that is advantageously higher than that of native human or pig blood.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells; and (iv) subjecting the released contents of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin. In a preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

4. DESCRIPTION OF THE FIGURES

Figure 1. Recombinant nucleic acid constructs.

A. Construct ααβ (the "116 construct); B. Construct αpβ (the "185" construct); C. Construct βpα (the "290" construct); D. Construct εpζβα; E. Construct ζpεαpβ; F. Construct αpβ carrying a β108 Asn -> Asp mutation (the "hemoglobin Yoshizuka construct"); G. Construct αpβ carrying a β108 Asn -> Lys mutation (the "hemoglobin Presbyterian construct"); H. Construct αpβ(Δα) coinjected with LCR α (the "285" construct); I.

Construct αpβ carrying an α134 Thr -> Cys mutation (the "227" construct); J. Construct αpβ carrying an α104 Cys-> Ser mutation (the "227" construct), a β93 Cys -> Ala mutation, and a β112 Cys -> Val mutation (the "228" construct); K. Construct αpδ (the "263" construct); and L. Construct αpδ(Δα) coinjected with LCR α (the "274" construct); M. Construct LCR α coinjected with LCR εβ (the "240" construct); N. Construct αpβ carrying a β61 Lys -> Met mutation (the "Hemoglobin Bologna" construct); O. Construct LCR εαβ (the "318" construct); P. Construct LCR αεβ (the "319" construct); Q. Construct LCR ααεβ (the "329" construct); R. Construct LCR αε(^{pig}βp)β (the "339" construct); S. Construct αpβ carrying an α75 Asp -> Cys mutation (the "340" construct); T. Construct αpβ carrying an α42 Tyr -> Arg mutation (the "341" construct); U. Construct LCR εβαα (the "343" construct); V. Construct LCR εβα (the "347" construct); W. Construct αpβ carrying an α42 Tyr -> Lys mutation; X. Construct αpβ carrying an α42 Tyr -> Arg mutation; and a β99 Asp -> Glu mutation; Y. Construct αpβ carrying an α42 Tyr -> Lys mutation; and a β99 Asp -> Glu mutation.

Figure 2. Transgenic pig.

Figure 3. Demonstration of human hemoglobin expression in transgenic pigs. A. Isoelectric focusing gel analysis. B. Triton-acid urea gel of hemolysates of red blood cells representing human blood (lane 1); blood from transgenic pig 12-1 (lane 2), 9-3 (lane 3), and 6-3 (lane 4); and pig blood (lane 5) shows under-expression of human β globin relative to human α globin in the transgenic animals.

Figure 4. Separation of human hemoglobin and pig hemoglobin by DEAE chromatography. A. Hemolyzed mixture of human and pig red blood cells; B. Hemolysate of red blood cells collected from transgenic pig 6-3. C. Human and mouse hemoglobin do not separate by DEAE chromatography under these conditions. D. Isoelectric focusing of human hemoglobin purified from pig hemoglobin.

Figure 5. Isoelectric focussing gel of reassociated pig hemoglobin (lane 1); reassociated pig/human hemoglobin mixture (lanes 2 and 4); reassociated human hemoglobin (lane 3); and transgenic pig hemoglobin (lane 5).

Figure 6. Separation of human hemoglobin by QCIP chromatography.

Figure 7. Oxygen affinity of transgenic hemoglobin.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a method of producing human hemoglobin that utilizes transgenic pigs, novel globin-encoding nucleic acid constructs, and transgenic pigs that express human hemoglobin. For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) preparation of globin gene constructs;
(ii) preparation of transgenic pigs;
(iii) preparation of human hemoglobin and its separation from pig hemoglobin; and
(iv) preparation of human/pig hybrid hemoglobin.

5.1. PREPARATION OF GLOBIN GENE CONSTRUCTS

The present invention provides for a method of producing human globin and/or hemoglobin in transgenic pigs. Human hemoglobin is defined herein to refer to hemoglobin formed by globin chains encoded human globin genes (including alpha, beta, delta, gamma, epsilon and zeta genes) or variants thereof which are naturally occurring or the products of genetic engineering. Such variants are at least about ninety percent homologous in amino acid sequence to a naturally occurring human hemoglobin. In preferred embodiments, the human hemoglobin of the invention comprises a human alpha globin and a human beta globin chain. The human hemoglobin of the invention comprises at least two different globin chains, but may comprise more than two chains, to form, for example, a tetrameric molecule, octameric molecule, etc. In preferred embodiments of the invention, human hemoglobin consists of two human alpha globin chains and two human beta globin chains. As discussed infra, the present invention also provides for hybrid hemoglobin comprising human α globin and pig β globin.

According to particular embodiments of the present invention, at least one human globin gene, such as a human alpha and/or a human beta globin gene, under the control of a suitable promoter or promoters, is inserted into the genetic material of a pig so as to create a transgenic pig that carries human globin in at least some of its red blood cells. This requires the preparation of appropriate recombinant nucleic acid sequences. In preferred embodiments of the invention, both human α and human β genes are expressed. In an alternative embodiment, only human α globin is expressed. In further embodiments, human

embryonic or fetal globin genes are expressed or are used as developmental expression regulators of adult genes.

Human alpha and beta globin genes may be obtained from publicly available clones, e.g. as described in Swanson et al., 1992, Bio/Technol. 10:557-559. Nucleic acid sequences encoding human alpha and beta globin proteins may be introduced into an animal via two different species of recombinant constructs, one which encodes human alpha globin, the other encoding human beta globin; alternatively, and preferably, both alpha and beta-encoding sequences may be comprised in the same recombinant construct.

A suitable promoter, according to the invention, is a promoter which can direct transcription of human alpha and beta globin genes in red blood cells. Such a promoter is preferably selectively active in erythroid cells. This would include, but is not limited to, a globin gene promoter, such as the human alpha, beta, delta, epsilon or zeta promoters, or a globin promoter from another species. It may, for example, be useful to utilize pig globin promoter sequences. The human alpha and beta globin genes may be placed under the control of different promoters, but, since it has been inferred that vastly different levels of globin chain production may result in lethality, it may be preferable to place the human alpha and beta globin genes under the control of the same promoter sequence. In order to avoid chain imbalance and/or titration of transcription factors due to constitutive β-globin promoter activity in an inappropriate cell type, it is desirable to design a construct which leads to coordinate expression of human alpha and beta globin

genes at the same time in development and at quantitatively similar levels.

In one particular, non-limiting embodiment of the invention, a construct comprising the $\alpha\alpha\beta$ construct (also termed the "116" construct; Swanson et al., 1992, Bio/Technol. 10:557-559; see Figure 2A) may be utilized. Although this construct, when present as a transgene at high copy number, has resulted in deleterious effects in mice, it has been used to produce healthy transgenic pigs (see Example Section 6, infra).

In another particular, non-limiting embodiment of the invention, a construct comprising the $\alpha p\beta$ sequence (also termed the "185" construct), as depicted in Figure 1B may be used. Such a construct has the advantage of placing both alpha and beta globin-encoding sequences under the control of the same promoter (the alpha globin promoter).

The present invention, in further specific embodiments, provides for (i) the construct $\beta p\alpha$, in which the human alpha and beta globin genes are driven by separate copies of the human beta globin promoter (Figure 1C); (ii) the $\epsilon p\zeta\beta p\alpha$ construct, which comprises human embryonic genes zeta and epsilon under the control of the epsilon promoter and both alpha and beta genes under the control of the beta promoter (Figure 1D); (iii) the $\zeta p\epsilon\alpha p\beta$ construct, which comprises human embryonic genes zeta and epsilon under the control of the zeta promoter and both alpha and beta genes under the control of the alpha promoter (Figure 1E); (iv) the $\alpha p\beta$ construct carrying a mutation that results in an aspartic acid residue (rather than an asparagine residue) at amino acid number 108 of β globin protein, to produce hemoglobin Yoshizuka (Figure 1F); (v) the $\alpha p\beta$ construct carrying

a mutation that results in a lysine residue (rather than an asparagine residue) at amino acid number 108 of β-globin protein, to produce hemoglobin Presbyterian (Figure 1G); (vi) the αpβ(Δα) construct, coinjected with LCR α which comprises the human β-globin gene under the control of the human α-globin promoter and a separate nucleic acid fragment comprising the human α-globin gene under its own promoter (Figure 1H); (vii) the αpβ construct carrying a mutation that results in a cysteine residue (rather than a threonine residue) at amino acid number 134 of α-globin protein (Figure 1I); (viii) the αpβ construct carrying a mutation that results in a serine residue (rather than a cysteine residue) at amino acid number 104 of the α-globin protein, an alanine residue (rather than a cysteine residue) at amino acid number 93 of the β-globin protein and a valine residue (rather than a cysteine residue) at amino acid number 112 of the β-globin protein (Figure 1J); (ix) the αpδ construct, which comprises the human adult α-globin promoter under its own promoter and the human δ-globin gene under the control of the human adult α-globin promoter (Fig. 1K); (x) Construct αpδ(Δα) coinjected with LCR α, which comprises the human δ-globin gene under the control of the human α-globin promoter and a separate nucleic acid fragment comprising the human α-globin gene under its own promoter (Fig. 1L); (xi) Construct LCR α coinjected with LCR εβ, which comprises the human α-globin gene under the control of its own promoter and a separate nucleic acid fragment comprising the human embryonic ε-globin gene and the adult β-globin gene under the control of their own promoters (Fig. 1M); (xii) the αpβ construct carrying a mutation that results in a methionine residue (rather than a lysine residue) at amino acid number 61

of the α-globin protein (Fig. 1N); (xiii) the εαβ construct, which comprises the human embryonic epsilon gene, the human adult alpha globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1O); (xiv) the αεβ construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1P); (xv) the ααεβ construct, which comprises two copies of the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1Q); (xvi) the αε(pigβp)β construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene under the control of the endogenous porcine adult beta globin promoter all linked in tandem from 5'- to 3' (Fig. 1R); (xvii) the αpβ construct carrying a mutation that results in a cysteine residue (rather than an aspartic acid residue) at amino acid number 75 of the α-globin protein (Fig. 1S); (xviii) the αpβ construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the α-globin protein (Fig. 1T); (xvix) the LCR εβαα construct, which comprises the human embryonic epsilon globin gene, the human adult beta globin gene and two copies of the human adult alpha-globin gene linked in tandem from 5'- to 3' (Fig. 1U); (xx) the LCR εβα construct, which comprises the human embryonic epsilon globin gene, the human adult beta globin gene and the human adult alpha-globin gene linked in tandem from 5'- to 3' (Fig. 1V); (xxi) the αpβ construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the α-

globin protein (Fig. 1W); (xxii) the αpβ construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the α-globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the β-globin protein (Fig. 1X); and (xxiii) the αpβ construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the α-globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the β-globin protein (Fig. 1Y).

The recombinant nucleic acid constructs described above may be inserted into any suitable plasmid, bacteriophage, or viral vector for amplification, and may thereby be propagated using methods known in the art, such as those described in Maniatis et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. In the working examples presented below, the PUC vector (Yanish-Perron et al., 1985, Gene 103-119) was utilized.

Constructs may desirably be linearized for preparation of transgenic pigs. Vector sequence may desirably be removed.

5.2. PREPARATION OF TRANSGENIC PIGS

The recombinant constructs described above may be used to produce a transgenic pig by any method known in the art, including but not limited to, microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection, transduction, retroviral infection, etc. Species of constructs may be introduced individually or in groups of two or more types of construct.

According to a preferred specific embodiment of the invention, a transgenic pig may be produced by the methods as set forth in Example Section 6, infra. Briefly, estrus may be synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts may be given an intramuscular injection (IM) of prostaglandin $F_{2\alpha}$ (Lutalyse: 10 mg/injection) at 0800 and 1600 hours. Twenty-four hours after the last day of AT consumption all donor gilts may be administered a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) may be administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts may be checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration may be bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG one- and two-cell ova may be surgically recovered from bred donors using the following procedure. General anesthesia may be induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract may be exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) may be inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova may be flushed in retrograde fashion by inserting a 20 g needle into the

lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) may be infused into the oviduct and flushed toward the glass cannula. The medium may be collected into sterile 17 x 100 mm polystyrene tubes. Flushings may be transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova may be washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 µl drops of BMOC-3 medium under oil. Ova may be stored at 38°C under a 90% N_2, 5% O_2, 5% CO_2 atmosphere until microinjection is performed.

One- and two-cell ova may be placed in a Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova may then be transferred to a 5 - 10 µl drop of HEPES medium under oil on a depression slide. Microinjection may be performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (linearized at a concentration of about 1ng/µl of Tris-EDTA buffer) may be injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova may be returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N_2, 5% CO_2, 5% O_2 atmosphere prior to their transfer to suitable recipients. Ova may preferably be transferred within 10 hours of recovery.

Only recipients which exhibit estrus on the same day or 24 hours later than the donors may preferably be utilized for embryo transfer.

Recipients may be anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one-and/or two-cell ova and 4-6 control ova may be transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set may be connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium may be aspirated into the tubing. The tubing may then be fed through the ostium of the oviduct until the tip reaches the lower third or isthmus of the oviduct. The ova may be subsequently expelled as the tubing is slowly withdrawn.

The exposed portion of the reproductive tract may be bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin may be sutured as three separate layers. An uninterrupted Halstead stitch may be used to close the lina alba. The fat and skin may be closed using a simple continuous and mattress stitch, respectively. A topical antibacterial agent (e.g. Furazolidone) may then be administered to the incision area.

Recipients may be penned in groups of about four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients may be checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection may be performed using ultrasound. On day 107 of gestation recipients may be transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing may be induced by the administration of prostaglandin F_{2a} (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients may be expected to farrow within 34 hours following PGF2a administration.

Twenty-four hours after birth, all piglets may be processed, i.e. ears notched, needle teeth clipped, 1 cc of iron dextran administered, etc. A tail biopsy and blood may also be obtained from each pig.

Pigs produced according to this method are described in Example Section 6, infra, and are depicted in Figure 2. Such pigs are healthy, do not appear to be anemic, and appear to grow at a rate comparable to that of their non-transgenic littermates. Such pigs may transmit the transgene to their offspring.

Pigs having certain characteristics may be especially useful for the production of human hemoglobin; such pigs, examples of which follow, represent preferred, non-limiting, specific embodiments of the invention.

According to one preferred specific embodiment of the invention, a transgenic pig contains at least twenty copies of a globin transgene.

According to a second preferred specific embodiment, the P_{50} of whole blood of a transgenic pig according to the invention is increased by at least ten percent over the P_{50} of the whole blood of a comparable non-transgenic pig,taking into consideration factors such as altitude, oxygen concentrations, pregnancy, the presence of mutant hemoglobin, etc. Thus, the present invention provides for a non-pregnant transgenic pig that carries and expresses a human globin transgene in which the P_{50} of whole blood of the transgenic pig is at least ten percent greater than the P_{50} of whole blood of a comparable non-pregnant non-transgenic pig at the same altitude.

In other preferred specific embodiments, the present invention provides for a transgenic pig in which the amount of human globin produced relative to total hemoglobin is at least two percent, more preferably at least five percent, and most preferably at least ten percent.

Section 6, infra, describes transgenic pigs which serve as working examples of preferred, non-limiting, specific examples of the invention.

5.3. PREPARATION OF HUMAN HEMOGLOBIN AND ITS SEPARATION FROM PIG HEMOGLOBIN

The present invention provides for a method for producing human hemoglobin comprising introducing a transgene or transgenes encoding human hemoglobin, such as a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its blood cells.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells to form a lysate; (iv) subjecting the lysate of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin; and (v) collecting the fractions that contain purified human hemoglobin. Such fractions may be identified by isoelectric focusing in parallel with appropriate standards. In a

preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

In order to prepare human hemoglobin from the transgenic pigs described above, red blood cells are obtained from the pig using any method known in the art. The red blood cells are then lysed using any method, including hemolysis in a hypotonic solution such as distilled water, or using techniques as described in 1981, Methods in Enzymology Vol. 76, and/or tangential flow filtration.

For purposes of ascertaining whether human hemoglobin is being produced by a particular transgenic pig, it may be useful to perform a small-scale electrophoretic analysis of the hemolysate, such as, for example, isoelectric focusing using standard techniques.

Alternatively, or for larger scale purification, human hemoglobin may be separated from pig hemoglobin using ion exchange chromatography. Surprisingly, as discussed in Section 7, supra, human hemoglobin was observed to readily separate from pig hemoglobin using ion exchange chromatography whereas mouse hemoglobin and human hemoglobin were not separable by such methods. Any ion exchange resin known in the art or to be developed may be utilized, including, but not limited to, resins comprising diethylaminoethyl, Q-Sepharose, QCPI (I.B.F.) Zephyr, Spherodex, ectiola, carboxymethylcellulose, etc. provided that the resin results in a separation of human and pig hemoglobin comparable to that achieved using DEAE resin.

According to a specific, nonlimiting embodiment of the invention, in order to separate human from pig hemoglobin (including human/pig

hemoglobin hybrids) to produce substantially pure human hemoglobin, a hemolysate of transgenic pig red blood cells, prepared as above may be applied to a DEAE anion exchange column equilibrated with 0.2 M glycine buffer at pH 7.8 and washed with 0.2 M glycine pH 7.8/5 mM NaCl, and may then be eluted with a 5-30 mM NaCl gradient, or its equivalent (see, for example, Section 9 infra). Surprisingly, despite about 85 percent homology between human and pig globin chains, human and pig hemoglobin separates readily upon such treatment, with human hemoglobin eluting earlier than pig hemoglobin. Elution may be monitored by optical density at 405 nm and/or electrophoresis of aliquots taken from serial fractions. Pig hemoglobin, as well as tetrameric hemoglobin composed of heterodimers formed between pig and human globin chains, may be separated from human hemoglobin by this method. Human hemoglobin produced in a transgenic pig and separated from pig hemoglobin by this method has an oxygen binding capability similar to that of native human hemoglobin.

According to another specific, non-limiting embodiment of the invention, human hemoglobin may be separated from pig hemoglobin (including human/pig hemoglobin hybrids) using QCPI ion exchange resin as follows:

About 10 mg of hemoglobin prepared from transgenic pig erythrocytes may be diluted in 20ml of Buffer A (Buffer A = 10mM Tris, 20mM Glycine pH 7.5). This 20ml sample may then be loaded at a flow rate of about 5ml/min onto a QCPI column (10 ml) which has been equilibrated with Buffer A. The column may then be washed with 2 volumes of Buffer A, and then with 20 column volumes of a 0-50mM NaCl gradient (10 column volumes of Buffer A + 10 column volumes of 10mM Tris,

20mM Glycine, 50mM NaCl Ph 7.5) or, alteratively, 6 column volumes of 10mM Tris, 20mM Glycine, 15mM NaCl, pH 7.5, and the $O.D._{280}$ absorbing material may be collected in fractions to yield the separated hemoglobin, human hemoglobin being identified, for example, by isoelectric focusing using appropriate standards. The QCPI column may be cleaned by elution with 2 column volumes of 10mM Tris, 20mM Glycine, 1M NaCl, pH 7.5.

5.4. PREPARATION OF HUMAN/PIG HYBRID HEMOGLOBIN

The present invention also provides for essentially purified and isolated human/pig hybrid hemoglobin, in particular human α/pig β hybrid hemoglobin. Pig α/human β hybrid has not been observed to form either *in vitro* in reassociation experiments or *in vitro* in transgenic pigs.

The present invention provides for hybrid hemoglobin and its use as a blood substitute, and for a pharmaceutical composition comprising the essentially purified and isolated human/pig hemoglobin hybrid in a suitable pharmacological carrier.

Hybrid hemoglobin may be prepared from transgenic pigs, as described herein, and then purified by chromatography, immunoprecipitation, or any other method known to the skilled artisan. The use of isoelectric focusing to separate out hemoglobin hybrid is shown in Figures 3 and 5.

Alternatively, hybrid hemoglobin may be prepared using nucleic acid constructs that comprise both human and pig globin sequences which may then be expressed in any suitable microorganism, cell, or transgenic animal. For example, a nucleic acid construct that comprises the human α and pig β globin genes under the control of a suitable promoter may be

expressed to result in hybrid hemoglobin. As a specific example, human α globin and pig β globin genes, under the control of cytomegalovirus promoter, may be transfected into a mammalian cell such as a COS cell, and hybrid hemoglobin may be harvested from such cells. Alternatively, such constructs may be expressed in yeast or bacteria.

It may be desirable to modify the hemoglobin hybrid so as to render it non-immunogenic, for example, by linkage with polyethylene glycol or by encapsulating the hemoglobin in a membrane, e.g. in a liposome.

6. EXAMPLE: GENERATION OF TRANSGENIC PIGS THAT PRODUCE HUMAN HEMOGLOBIN

6.1. MATERIALS AND METHODS

6.1.1. NUCLEIC ACID CONSTRUCTS

Constructs 116 (the ααβ construct), 185 (the αpβ construct), or 263 (the αpδ construct) were microinjected into pig ova as set forth below in order to produce transgenic pigs.

6.1.2. PRODUCTION OF TRANSGENIC PIGS

Estrus was synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts received an intramuscular injection (IM) of prostaglandin $F_{2\alpha}$ (Lutalyse: 10 mg/injection) at 0800 and 1600. Twenty-four hours after the last day of AT consumption all donor gilts received a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) was administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts were checked twice daily for signs of estrus

using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration were bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG, one- and two-cell ova were surgically recovered from bred donors using the following procedure. General anesthesia was induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract was exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) was inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova were flushed in retrograde fashion by inserting a 20 g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) was infused into the oviduct and flushed toward the glass cannula. The medium was collected into sterile 17 x 100 mm polystyrene tubes. Flushings were transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova were washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 µl drops of BMOC-3 medium under oil. Ova were stored at 38°C under a 90% N_2, 5% O_2, 5% CO_2 atmosphere until microinjection was performed.

One- and two-cell ova were placed in an Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei

in one-cell and nuclei in two-cell ova. Ova were then transferred to a 5 -10 μl drop of HEPES medium under oil on a depression slide. Microinjection was performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (1ng/μl of Tris-EDTA buffer) were injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova were returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N_2, 5% CO_2, 5% O_2 atmosphere prior to their transfer to suitable recipients. Ova were transferred within 10 hours of recovery.

Only recipients which exhibited estrus on the same day or 24 hours later than the donors were utilized for embryo transfer. Recipients were anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one- and/or two-cell ova and 4-6 control ova were transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set was connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium were aspirated into the tubing. The tubing was then fed through the ostium of the oviduct until the tip reached the lower third or isthmus of the oviduct. The ova were subsequently expelled as the tubing was slowly withdrawn.

The exposed portion of the reproductive tract was bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin were sutured as three separate layers. An uninterrupted Halstead stitch was used to close the lina alba. The fat and skin were closed using a simple continuous and mattress stitch, respectively.

A topical antibacterial agent (Furazolidone) was then administered to the incision area.

Recipients were penned in groups of four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients were checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection was performed using ultrasound. On day 107 of gestation recipients were transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing was induced by the administration of prostaglandin $F_{2\alpha}$ (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients farrowed within 34 hours following PGF2a administration.

Twenty-four hours after birth, all piglets were processed, i.e. ears were notched, needle teeth clipped, 1 cc of iron dextran was administered, etc. A tail biopsy and blood were also obtained from each pig.

6.2. RESULTS AND DISCUSSION

Of 3566 injected ova, thirteen transgenic pigs that expressed human hemoglobin were born, two of which died shortly after birth due to normal breeding-related incidents completely unrelated to the fact that they were transgenic pigs (Table I). The remaining 11 have appeared to be healthy. A photograph of one transgenic pig is presented in Figure 2. Profiles of the pigs and of the percent "authentic" and "hybrid" human hemoglobin ("HB") produced are set forth in Table II, infra. Total hemoglobin was calculated as the sum of human $\alpha\beta$ plus one-half of the human α pig β hybrid. Figure 3 presents the results of isoelectric focusing and

triton acid urea gels of hemoglobin produced by three of these pigs (numbers 12-1, 9-3, and 6-3) which demonstrate the expression of human alpha and beta globin in these animals.

TABLE I

Efficiency of Transgenic Pig Production
Human Hemoglobin Gene Construct(s)

Parameter	Total After 22 Trials
Total Ova Collected	8276
Total # Fertilized	7156
Total # Injected	3566
# Injected Ova Transferred	3566
# Control Ova Transferred	279
# Recipients Used	104
# Pigs Born (Male, Female)	208,332
# Transgenic (Male, Female)	8,5 (0.36)[a]
# Expressing	13

[a] Proportion of injected ova which developed into transgenic pigs (13 transgenics/3566 injected ova).

TABLE II

FOUNDERS

PIG	GENDER	TRANSGENE CONSTRUCT	AUTHENTIC HUMAN HB	HYBRID HB	TOTAL HUMAN HB	COPY #
6-3	F	116	6.2%	8.1%	10.3%	57
9-3	F	116	1.0%	33.1%	16.6%	1
22-2	M	185	<1%	5.0%	5.0%	55
33-7	F	185	*died shortly after birth			0.5
38-1	F	185	1.0%	8.3%	5.2%	17
38-3	M	185	4.7%	17.2%	13.2%	22
38-4	M	185	3.2%	7.0%	6.7%	5
47-3	M	263	<1%	2.9%	2.0%	4-6
47-4	F	263	<1%	18.5%	10.0%	1-2
52-3	M	263	<1%	7.6%	4.0%	
52-7	M	263	<1%	26.4%	13.0%	
53-11	M	263	<1%	15.5%	8.0%	

Table III presents the profiles of offspring of pig number 9-3, which shows that the F1 generation of transgenic pigs are capable of expressing hemoglobin. Of note, none of the offspring of pig number 6-3 were found to be transgenic, possibly due to the absence of transgene in the animal's reproductive tissue.

TABLE III

F1 (OFFSPRING) OF PIG 9-3

PIG	GENDER	CONST.	AUTHENTIC HUMAN HB	HYBRID HUMAN HB	TOTAL HUM.	COPY #
9-3-1	F	116	1.0%	31.5%	16.0%	1
9-3-2*	F	116	1.0%	32.9%	17.0%	1
9-3-3	M	116	1.0%	29.7%	15.0%	1
9-3-4	M	116	1.0%	32.8%	17.0%	1
9-3-6	F	116	1.0%	29.1%	15.0%	1
9-3-8	M	116	1.0%	31.6%	16.0%	1
9-3-9	M	116	1.0%	30.2%	16.0%	1

*9-3-2 died the day after birth.

The birth weights of the transgenic pigs have been approximately equivalent to the birth weights of their non-transgenic littermates. As the transgenic pigs matured, their weights remained comparable to the weights of control animals.

7. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY DEAE CHROMATOGRAPHY

7.1. MATERIALS AND METHODS

7.1.1. PURIFICATION BY DEAE CHROMATOGRAPHY

For purification, red blood cells were collected by centrifugation of 5000 rpm for 3 minutes in an eppendorf microcentrifuge and washed three times with an equal volume (original blood) of 0.9% NaCl. Red cells were lysed with 1.5 volumes deionized H_2O, centrifuged at 15,000 rpm, and the supernatant was fractionated by anion exchange chromatography. DEAE cellulose chromatography (DE-SE manufactured by Whatman, Ltd.) was performed according to W. A. Schroeder and T. H. J. Huisman "The Chromatography of Hemoglobin", Dekker, New York, pp. 74-77. The 0.25 ml red cell hemolysate described above was applied to 1 cm x 7 cm DE-52 column pre-equilibrated in 0.2 M glycine pH 7.8 and was washed with 5 column volumes of 0.2 M glycine pH 7.8/5 mM NaCl. Hemoglobins were eluted with a 200 ml 5-30 mM NaCl/0.2 M glycine pH 7.8 gradient. To complete elution of pig hemoglobin, an additional 50 to 100 ml of 30 mM CaCl/glycine pH 7.8 was added to the column. Elution of hemoglobin was monitored by absorbance of 415 mM and by IEF analysis of column fractions.

7.1.2. REASSOCIATION OF GLOBIN CHAINS

Reassociation of globin chains was performed essentially as described in Methods in Enzymol.

76:126-133. 25 lambda of pig blood, 25 lambda of human blood,or a 25 lambda mixture of 12.5 lambda human blood and 12.5 lambda pig blood were treated as follows. The blood was pelleted at a setting of 5 on microfuge for 2 minutes, then washed three times with 100 lambda 0.9 percent NaCl. The cells were lysed with 50 lambda H_2O, then spun at high speed to confirm lysis. 50 lambda of the lysed cells was then combined with 50 lambda 0.2 M Na Acetate, pH 4.5, put on ice and then incubated in a cold room overnight. After adding 1.9 ml 0.1 M $NaH_2PO_4$4, pH 7.4 each sample was spun in centricon tubes at 4°C and 5K until about 0.5 ml remained. Then 1 ml of 0.1 M NaH_2PO_4 pH 7.4 was added and spun through at about 5K until about 0.2 ml volume was left. The hemoglobin was then washed from the walls of the centricon tube, an eppendorf adaptor was attached, and a table top microfuge was used to remove each sample from its centricon tube. The samples were then analyzed by isoelectric focusing.

7.2. RESULTS AND DISCUSSION

7.2.1. HUMAN AND PIG HEMOGLOBIN WERE SEPARATED FROM A HEMOLYZED MIXTURE OF HUMAN AND PIG BLOOD

Equal proportions of human and of pig blood were mixed and lysed, and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4A, pig hemoglobin separated virtually completely from human hemoglobin. This complete separation is surprising in light of the structural similarity between human and pig hemoglobin; pig and human alpha globin chains are 84.4 percent homologous and pig and human beta globin chains are 84.9 percent homologous. It is further surprising because, as shown in Figure 4C, when human and mouse blood was mixed, hemolyzed, applied to and

eluted from a DEAE column according to methods set forth in Section 7.1.1., supra, human and mouse hemoglobin were not observed to separate despite the fact that mouse and human alpha globin chains are about 85.8 percent homologous and mouse and human beta globin chains are 80.1 percent homologous. The ease of separation of human and pig hemoglobin on DEAE resin appears to be both efficient and economical.

Interestingly, the order of elution of the proteins from the anion exchange column was not as expected. Based on the relative pI's of the proteins as deduced from the IEF gels, the predicted order of elution would be first the hybrid (human α/pig β) followed by the authentic human α/human β. The last protein to elute from the anion exchange column then would be the endogenous pig α/pig β protein. However, under all the conditions currently attempted the order of elution was altered such that the human hemoglobin was the first to elute. The second peak was an enriched fraction of the hybrid followed very closely by the pig hemoglobin.

7.2.2. HUMAN AND PIG HEMOGLOBIN AND HUMAN/PIG HETEROLOGOUS HEMOGLOBIN WERE SEPARATED FROM HEMOLYSATE PREPARED FROM A TRANSGENIC PIG

Blood from transgenic pig 6-3 (as described in Section 6, supra) was lysed by hypotonic swelling and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4B, human hemoglobin was separated from pig hemoglobin and from human α globin/pig beta globin heterologous hemoglobin. As shown in Figure 4D, human hemoglobin was substantially purified by this method.

7.2.3. PIG ALPHA GLOBIN/HUMAN BETA GLOBIN HETEROLOGOUS HEMOGLOBIN DOES NOT APPEAR TO FORM BASED ON REASSOCIATION DATA

Heterologous association between pig alpha globin and human beta globin chains has not been detected in hemolysates obtained from human hemoglobin-expressing transgenic pigs. It was possible, however, that this observation could be explained by relatively low levels of human beta globin expression. Alternatively, association between pig alpha globin and human beta globin may be chemically unfavorable. In order to explore this possibility, reassociation experiments were performed in which pig and human hemoglobin were mixed, dissociated, and then the globin chains were allowed to reassociate. As shown in the isoelectric focusing gels depicted in Figure 5, although pig α/pig β, human α/human β, and human α/pig β association was observed, no association between pig α globin and human β globin appeared to have occurred. Therefore the pig α/human β heterologous hemoglobin should not be expected to complicate the purification of human hemoglobin from transgenic pigs.

8. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY QCPI CHROMATOGRAPHY

8.1. MATERIALS AND METHODS

Clarified hemolysate from transgenic pig 6-3 13mg/ml; Buffer A: 10mM Tris, 20mM Glycine pH 7.5; Buffer B: 10mM Tris, 20mM Glycine, 15 mM NaCl pH 7.5; Buffer C: 10mM Tris, 20mM Glycine, 1M NaCl pH 7.5; Buffer D: 10mM Tris, 20mM Glycine, 50 mM NaCl pH 7.5; QCPI column 10ml Equilibrated in Buffer A; Trio purification system. 10mg of hemoglobin prepared from

transgenic pig 6-3 was diluted in 20ml Buffer A. 20ml of sample was loaded at a flow rate of 5ml/min onto the QCPI column, and washed with 2 column volumes of Buffer A. The column was then washed with 20 column volumes of a 0-50mM NaCl gradient. (10 column volumes Buffer A + 10 column volumes of Buffer D) and the $O.D._{280}$ absorbing material was collected. The column was then cleaned with 2 column volumes of Buffer C, and then re-equilibrated with 2 column volumes of Buffer A.

8.2. RESULTS

Analysis of the UV trace (peak vs. volume of gradient) (Fig. 6) revealed that the human hemoglobin was eluted at 15 mM NaCl. Subsequent purifications have been performed utilizing the same protocol as above, only using 6 column volumes of Buffer B (15mM NaCl) to elute the human hemoglobin rather than the gradient. In addition, non-transgenic pig chromatographed by this method does not elute from the QCPI with Buffer B, while native human hemoglobin does. The protein that eluted at 15mM NaCl was analyzed on the Resolve isoelectric focussing system and found to be essentially pure of contaminating pig hemoglobin or hybrid hemoglobin.

9. EXAMPLE: HUMAN ALPHA/PIG BETA GLOBIN HYBRID HEMOGLOBIN EXHIBIT INCREASED P_{50}

As shown in Tables II and III, supra, transgenic pigs of the invention were all found to produce significant amounts of human α/pig β globin hybrid hemoglobin (the pig α/human β hybrid was not observed). Significantly, pigs that expressed higher percentages of hybrid also appeared to exhibit elevated P_{50} values for their whole blood (Figure 7).

Various publications are cited herein which are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A transgenic pig that (i) comprises a nucleic acid construct encoding human α globin and (ii) expresses human α globin in at least some of its red blood cells.

2. A transgenic pig that (i) comprises a nucleic acid construct encoding human α globin and a nucleic acid construct encoding human β globin and (ii) expresses human α globin and human β globin in at least some of its red blood cells.

3. A transgenic pig that (i) comprises a nucleic acid construct encoding human α globin and human β globin and (ii) expresses human α globin and human β globin in at least some of its red blood cells.

4. The transgenic pig of claim 1 in which the nucleic acid construct is the LCR α construct.

5. The transgenic pig of claim 2 in which the nucleic acid constructs are the LCR α and LCR εβ constructs.

6. The transgenic pig of claim 3 in which the nucleic acid construct is the 116 construct as depicted in Figure 1A.

7. The transgenic pig of claim 3 in which the nucleic acid construct is the 185 construct as depicted in Figure 1B.

8. The transgenic pig of claim 3 in which the nucleic acid construct is the βpα construct as depicted in Figure 1C.

9. The transgenic pig of claim 3 in which the nucleic acid construct is the hemoglobin Yoshizuka construct as depicted in Figure 1F.

10. The transgenic pig of claim 3 in which the nucleic acid construct is the hemoglobin Presbyterian construct as depicted in Figure 1G.

11. The transgenic pig of claim 3 in which the nucleic acid construct is the αpβ(Δα) construct as depicted in Figure 1H.

12. The transgenic pig of claim 3 in which the nucleic acid construct is the 227 construct as depicted in Figure 1I.

13. The transgenic pig of claim 3 in which the nucleic acid construct is the 228 construct as depicted in Figure 1J.

14. The transgenic pig of claim 3 in which the Hemoglobin Bologna construct is the 228 construct as depicted in Figure 1N.

15. The transgenic pig of claim 3 in which the nucleic acid construct is the 318 construct as depicted in Figure 1O.

16. The transgenic pig of claim 3 in which the nucleic acid construct is the 319 construct as depicted in Figure 1P.

17. The transgenic pig of claim 3 in which the nucleic acid construct is the 329 construct as depicted in Figure 1Q.

18. The transgenic pig of claim 3 in which the nucleic acid construct is the 339 construct as depicted in Figure 1R.

19. The transgenic pig of claim 3 in which the nucleic acid construct is the 340 construct as depicted in Figure 1S.

20. The transgenic pig of claim 3 in which the nucleic acid construct is the 341 construct as depicted in Figure 1T.

21. The transgenic pig of claim 3 in which the nucleic acid construct is the 343 construct as depicted in Figure 1U.

22. The transgenic pig of claim 3 in which the nucleic acid construct is the 347 construct as depicted in Figure 1V.

23. The transgenic pig of claim 3 in which the nucleic acid construct is as depicted in Figure 1W.

24. The transgenic pig of claim 3 in which the nucleic acid construct is as depicted in Figure 1X.

25. The transgenic pig of claim 3 in which the nucleic acid construct is as depicted in Figure 1Y6.

26. A transgenic pig that (i) comprises a nucleic acid construct encoding human δ globin and (ii) expresses human δ globin in at least some of its red blood cells.

27. The transgenic pig of claim 3 in which the nucleic acid construct is the 263 construct as depicted in Figure 1K.

28. The transgenic pig of claim 3 in which the nucleic acid construct is the 274 construct as depicted in Figure 1L.

29. The transgenic pig of claim 1, 2 or 3 which comprises, in a single cell, at least twenty copies of a globin transgene.

30. The transgenic pig of claim 1, 2 or 3 in which the P_{50} of the whole blood of the transgenic pig, when non-pregnant, is at least ten percent greater than the P_{50} of whole blood of a non-pregnant non-transgenic pig at the same altitude.

31. The transgenic pig of claim 1, 2 or 3 in which the amount of human globin produced relative to total hemoglobin is at least two percent.

32. The transgenic pig of claim 1, 2 or 3 in which the amount of human globin produced relative to total hemoglobin is at least five percent.

33. The transgenic pig of claim 1, 2 or 3 in which the amount of human globin produced relative to total hemoglobin is at least ten percent.

34. A method of purifying human hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

(i) collecting red blood cells from a transgenic pig according to claim 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25;

(ii) releasing the contents of the collected red blood cells to produce a lysate;

(iii) applying the lysate of step (ii) to a DEAE anion exchange column equilibrated with 0.2M glycine at a pH of 7.8;

(iv) eluting the column with a 5-30 mM NaCl gradient; and

(v) collecting the fractions that contain purified human hemoglobin.

35. A method of purifying human hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

(i) collecting red blood cells from a transgenic pig according to claim 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,16,17, 18, 19, 20, 21, 22, 23, 24, or 25;

(ii) releasing the contents of the collected red blood cells to produce a lysate;

(iii) applying the lysate of step (ii) to a QCIP column equilibrated with 10mM Tris, 20mM Glycine pH 5.0;

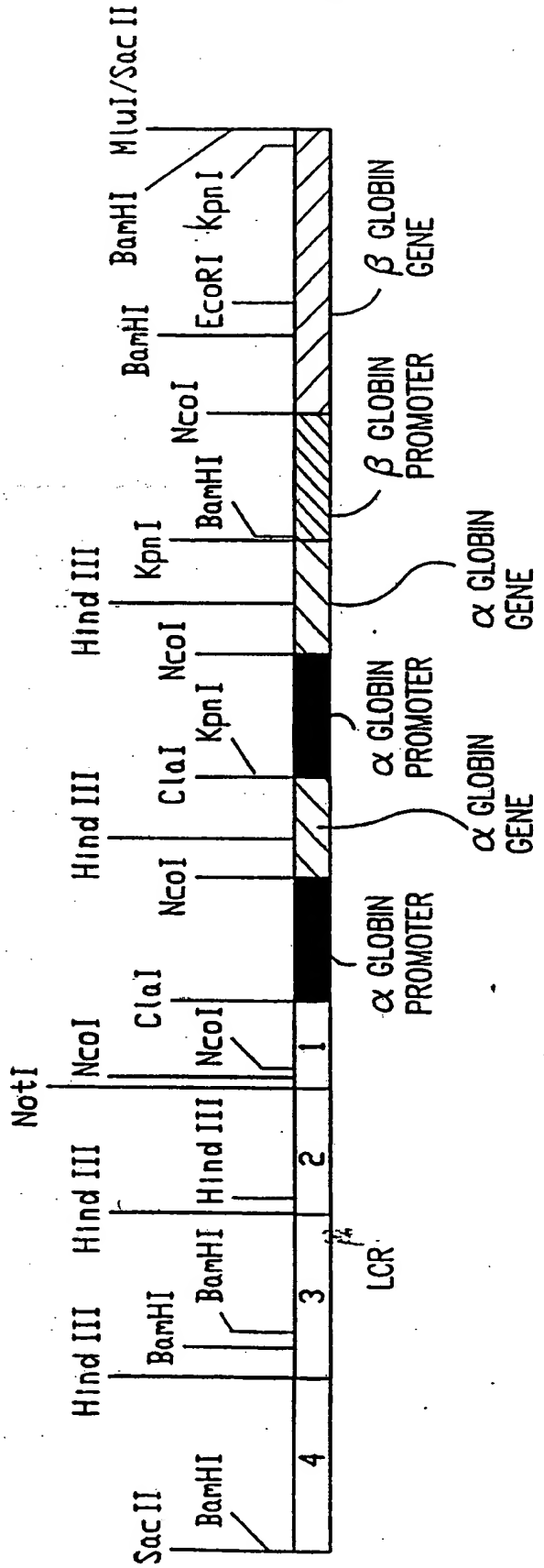
(iv) eluting the column with 6 column volumes of 10mM Tris, 20mM Glycine, 15mM NaCl, pH 7.5; and

(v) collecting the fractions that contain purified hemoglobin.

36. An essentially purified and isolated human/pig hemoglobin hybrid comprising human α globin and pig β globin.

37. A nucleic acid construct comprising a human α globin gene and a pig beta globin gene under the control of suitable promoter sequences.

38. A pharmaceutical composition comprising the essentially purified and isolated human/pig hemoglobin hybrid of claim 36 in a suitable pharmacological carrier.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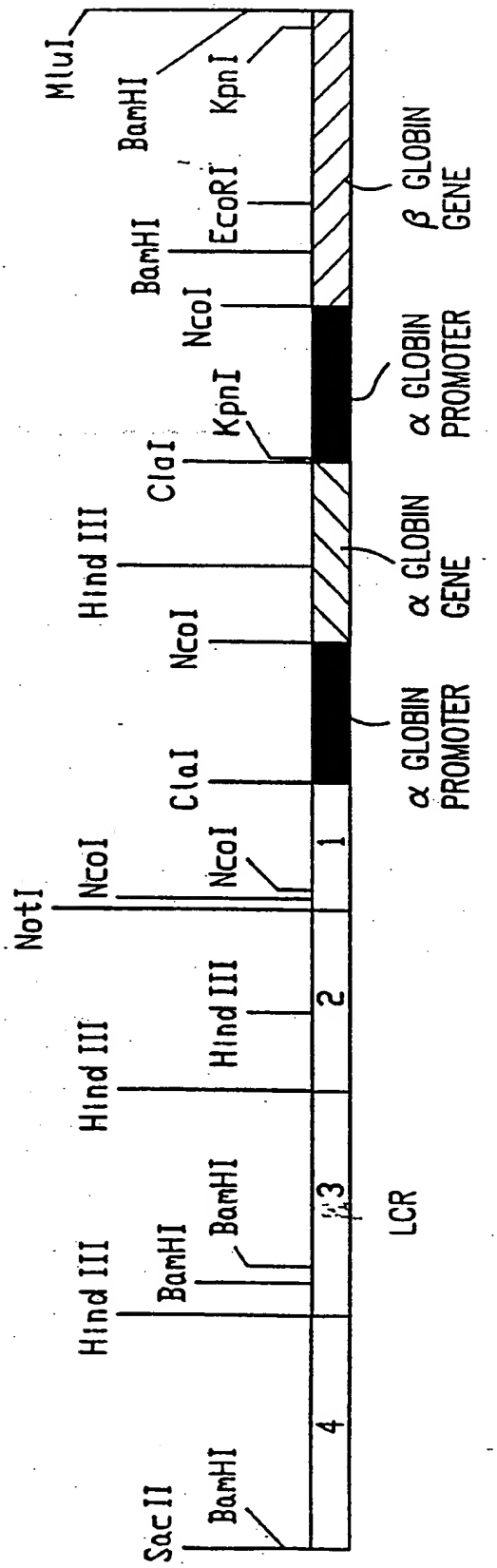
Hind III
BamHI
Hind III
BamHI
Hind III
NotI
NcoI
NcoI
ClaI
NcoI
Hind III
ClaI
KpnI
NcoI
Hind III
KpnI
BamHI
NcoI
BamHI
EcoRI
KpnI
BamHI
MluI/SacII
1
2
3
4
LCR
α GLOBIN PROMOTER
α GLOBIN GENE
α GLOBIN PROMOTER
α GLOBIN GENE
β GLOBIN PROMOTER
β GLOBIN GENE

FIG.1A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BamHI
KpnI
EcoRI
β GLOBIN GENE
BamHI
NcoI
α GLOBIN PROMOTER
KpnI
ClaI
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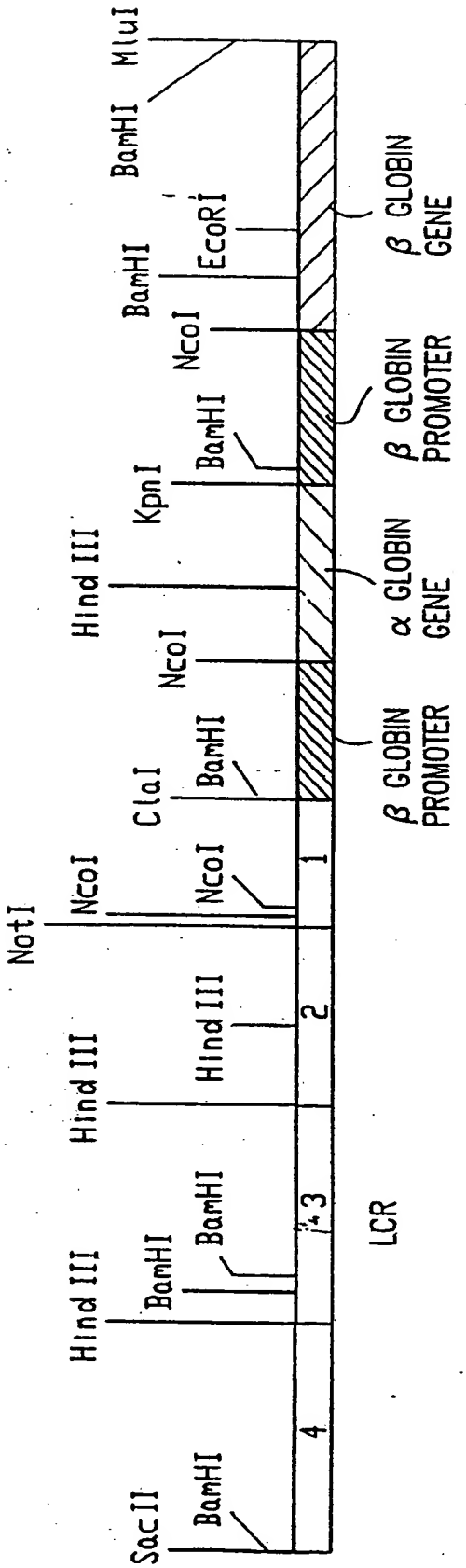
α GLOBIN GENE
NcoI
ClaI
α GLOBIN PROMOTER
NcoI
NcoI
NotI
1
Hind III
Hind III
2
BamHI
BamHI
Hind III
3
LCR
4
BamHI
SacII

FIG.1B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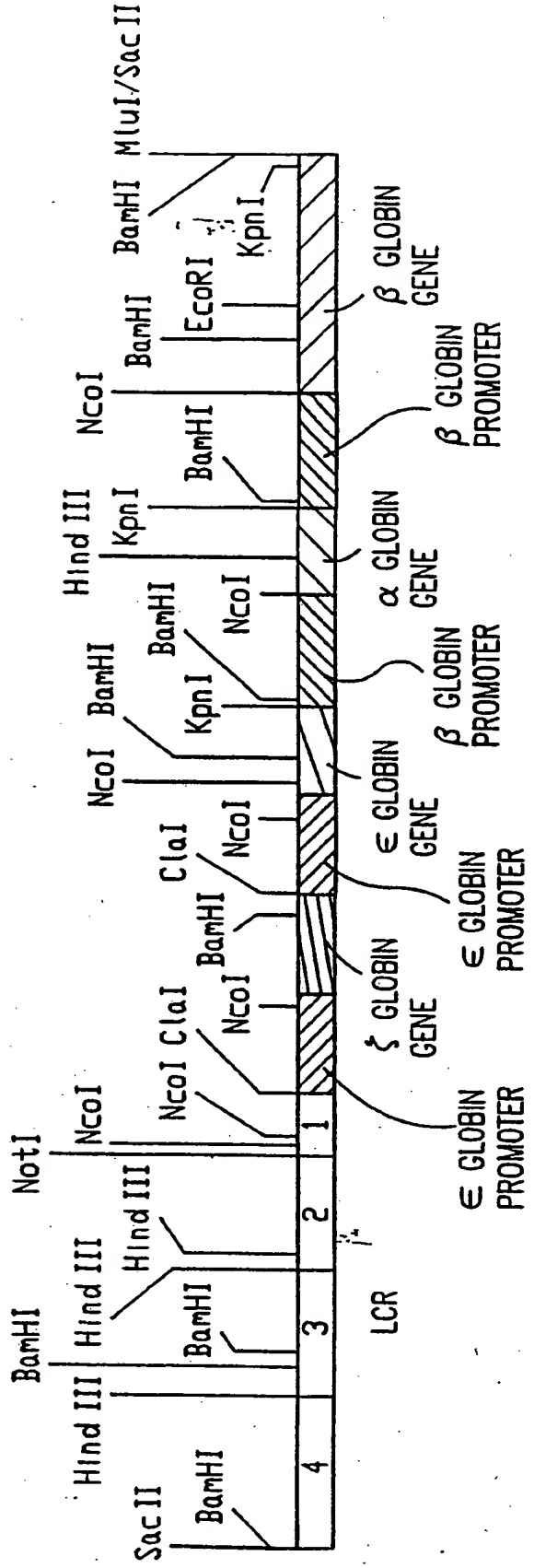
EcoRI
BamHI
NcoI
β GLOBIN PROMOTER
BamHI
KpnI
α GLOBIN GENE
Hind III
NcoI
β GLOBIN PROMOTER
BamHI
ClaI
NcoI
NotI
1
Hind III
2
Hind III
BamHI
3
LCR
BamHI
Hind III
4
BamHI
SacII

FIG. 1C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HindIII
BamHI
HindIII
BamHI
NotI
HindIII
NcoI
NcoI
ClaI
NcoI
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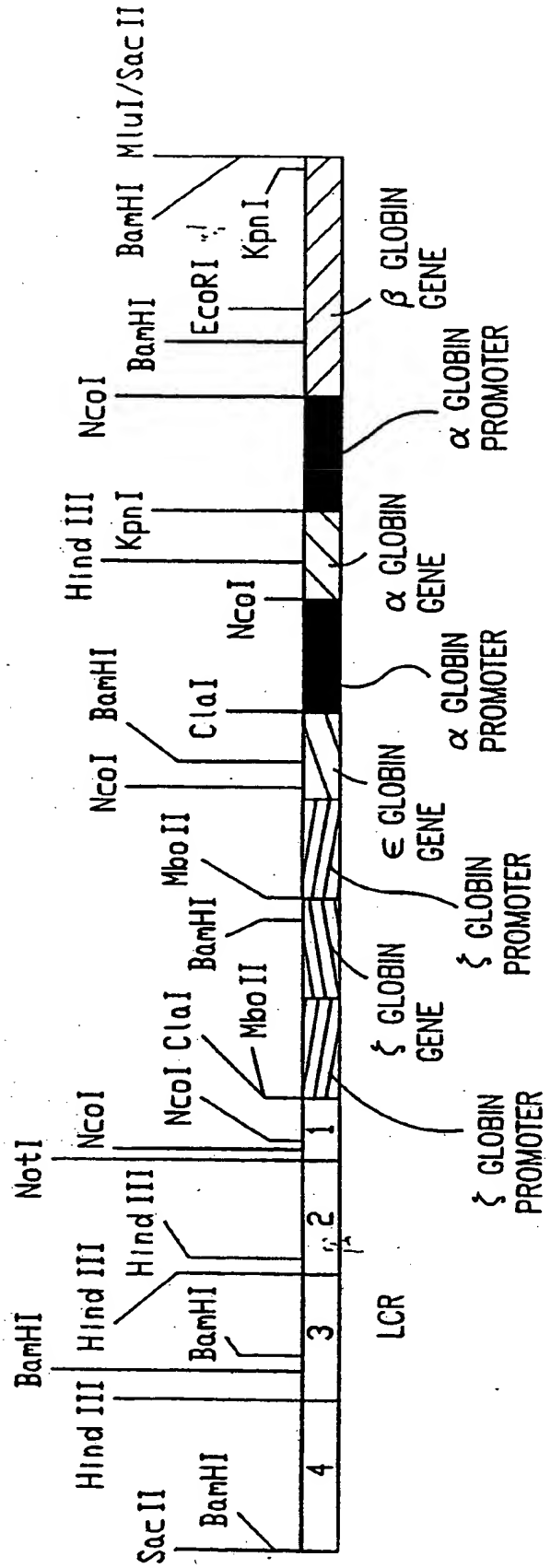
NcoI
ClaI
NcoI
BamHI
NcoI
BamHI
KpnI
HindIII
NcoI
BamHI
BamHI
EcoRI
KpnI
MluI/SacII
4
3
2
1
LCR
ε GLOBIN PROMOTER
ζ GLOBIN GENE
ε GLOBIN PROMOTER
ε GLOBIN GENE
β GLOBIN PROMOTER
α GLOBIN GENE
β GLOBIN PROMOTER
β GLOBIN GENE

FIG.1D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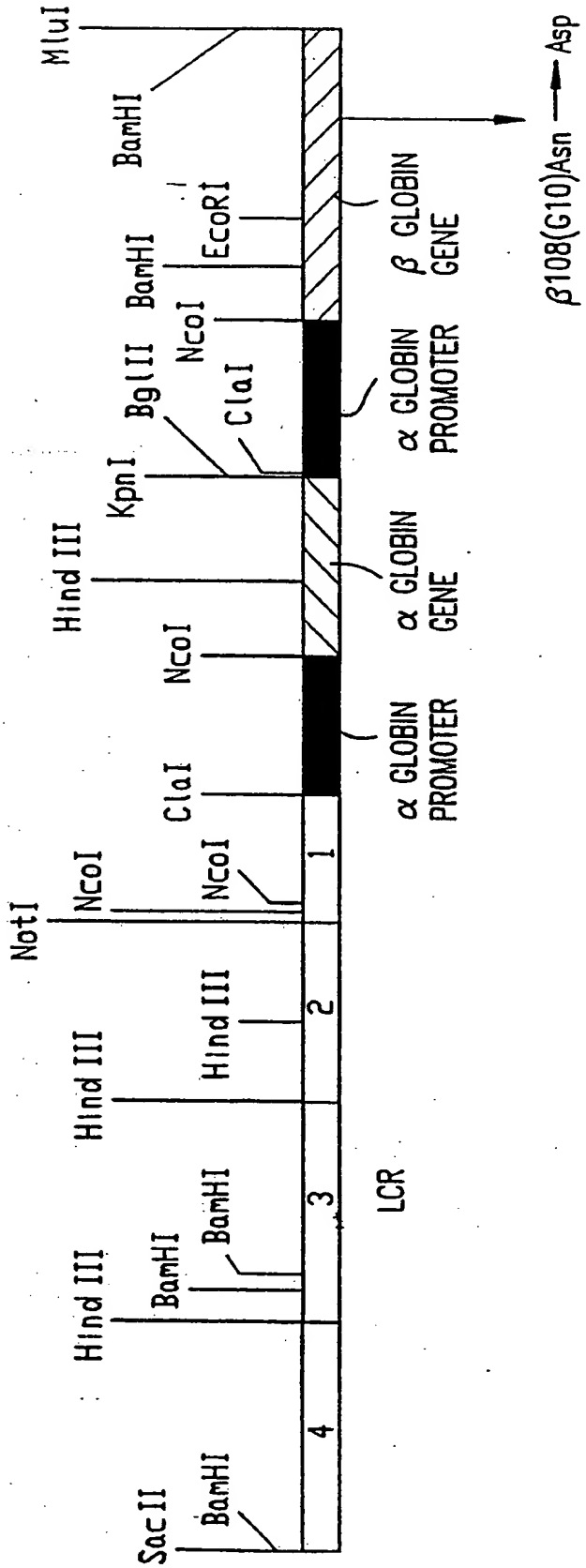
KpnI
EcoRI
BamHI
NcoI
β GLOBIN GENE
α GLOBIN PROMOTER
HindIII
KpnI
NcoI
α GLOBIN GENE
α GLOBIN PROMOTER
NcoI
BamHI
ClaI
ε GLOBIN GENE
ε GLOBIN PROMOTER
MboII
BamHI
ζ GLOBIN GENE
ζ GLOBIN PROMOTER
NcoI
ClaI
MboII
NotI
NcoI
HindIII
HindIII
BamHI
BamHI
HindIII
SacII
BamHI
1
2
3
4
LCR

FIG.1E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BamHI
NcoI
BglII
ClaI
KpnI
Hind III
NcoI
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
SacII
BamHI
1
2
3
4
LCR
α GLOBIN PROMOTER
α GLOBIN GENE
α GLOBIN PROMOTER
β GLOBIN GENE
β108(G10)Asn → Asp

FIG. 1F

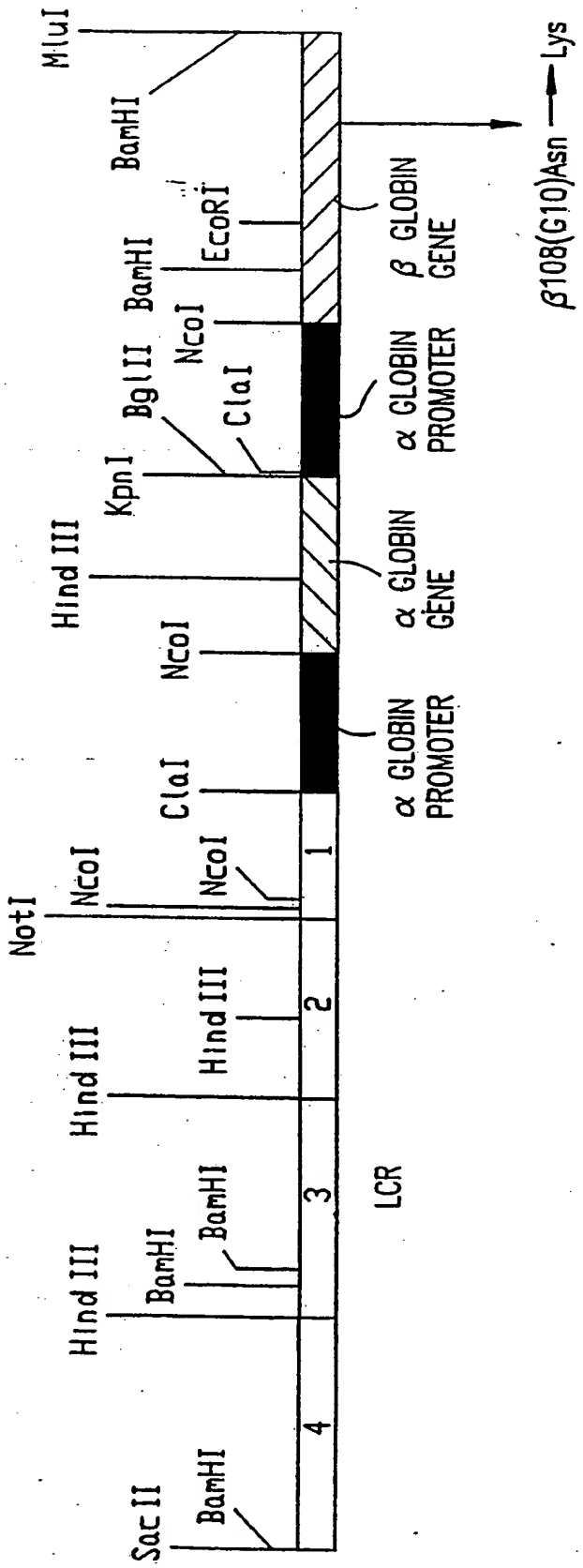
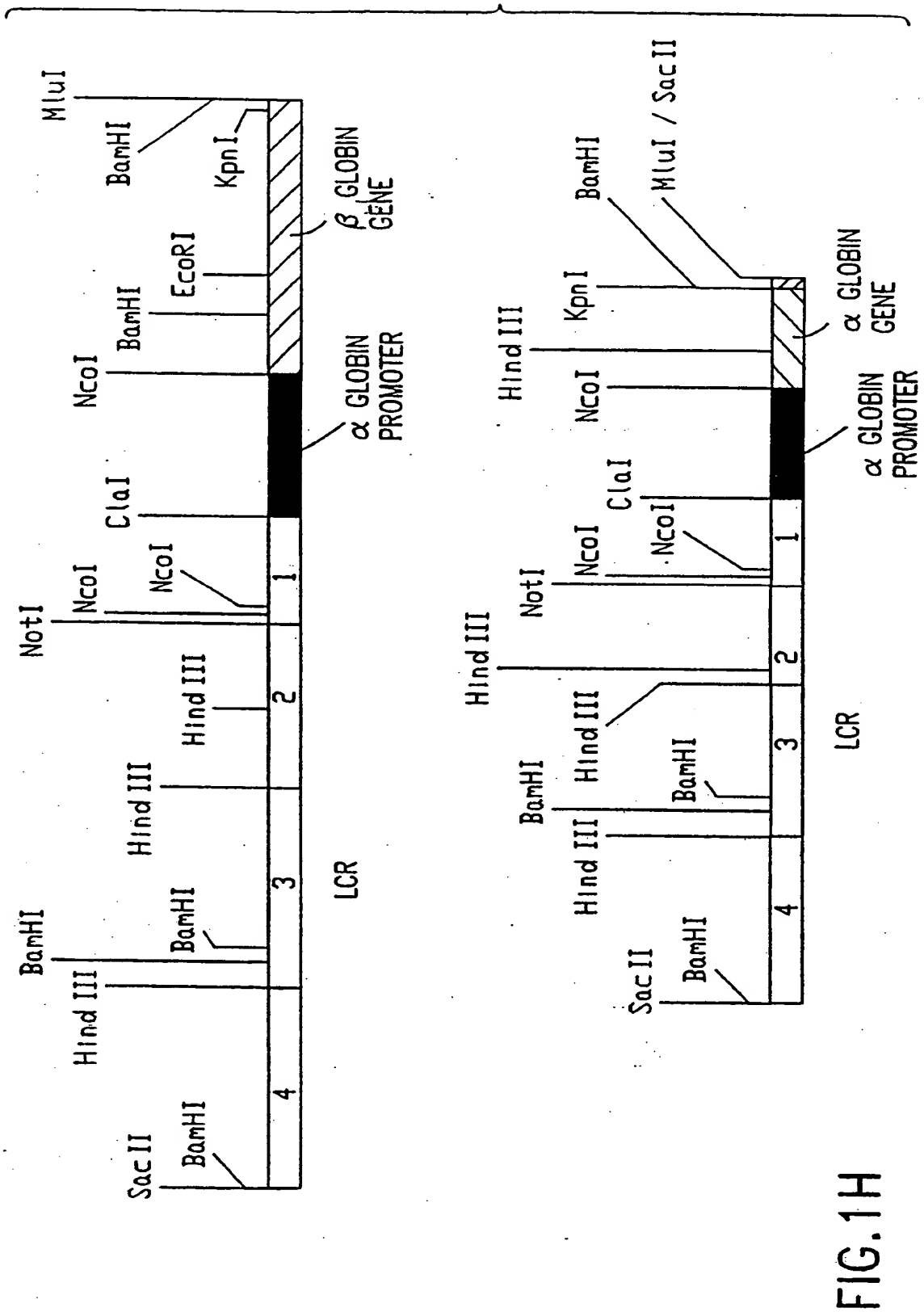

MluI
BamHI
EcoRI
BamHI
NcoI
BglII
ClaI
KpnI
Hind III
NcoI
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Sac II
1
2
3
4
LCR
α GLOBIN PROMOTER
α GLOBIN GENE
α GLOBIN PROMOTER
α GLOBIN PROMOTER
β GLOBIN GENE
β108(G10)Asn → Lys

FIG.1G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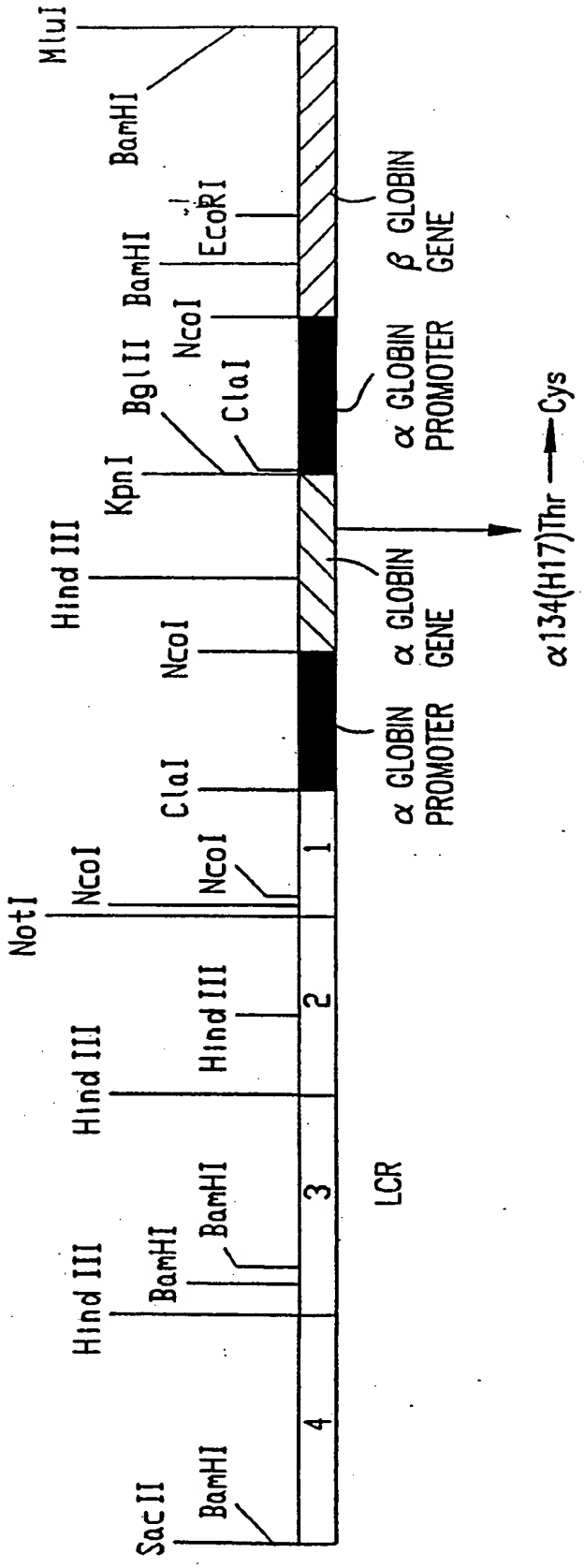
BamHI
KpnI
β GLOBIN GENE
EcoRI
BamHI
NcoI
α GLOBIN PROMOTER
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
Sac II
BamHI
LCR
1
2
3
4
Hind III
BamHI
MluI / Sac II
KpnI
α GLOBIN GENE
NcoI
α GLOBIN PROMOTER
ClaI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
Sac II
BamHI
LCR
1
2
3
4

FIG. 1H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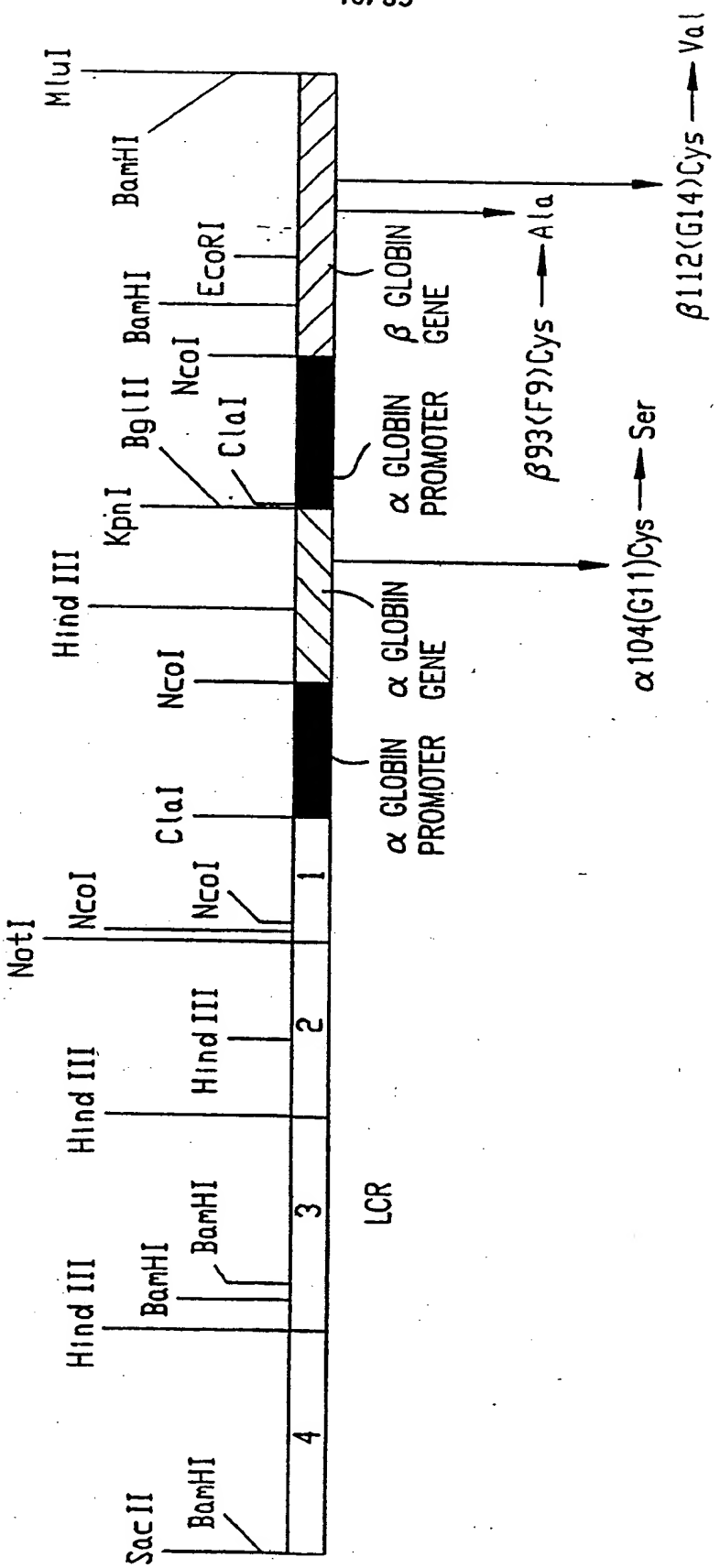
BamHI
EcoRI
BamHI
NcoI
BglII
ClaI
KpnI
Hind III
NcoI
ClaI
NcoI
NotI
NcoI
Hind III
Hind III
BamHI
BamHI
Hind III
SacII
BamHI
1
2
3
4
LCR
α GLOBIN PROMOTER
α GLOBIN GENE
α GLOBIN PROMOTER
α134(H17)Thr → Cys
β GLOBIN GENE
α GLOBIN PROMOTER

FIG. 11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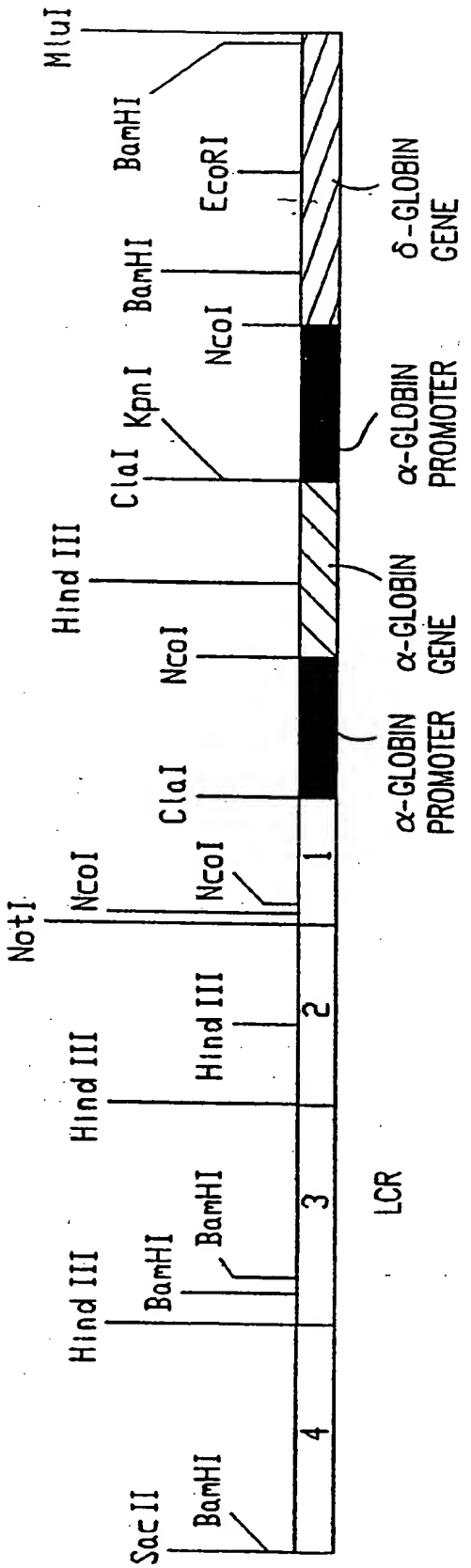
BamHI
EcoRI
BamHI
NcoI
Bgl II
ClaI
KpnI
Hind III
NcoI
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Sac II
BamHI
1
2
3
4
LCR
α GLOBIN PROMOTER
α GLOBIN GENE
α GLOBIN PROMOTER
β GLOBIN GENE
α104(G11)Cys → Ser
β93(F9)Cys → Ala
β112(G14)Cys → Val

FIG. 1J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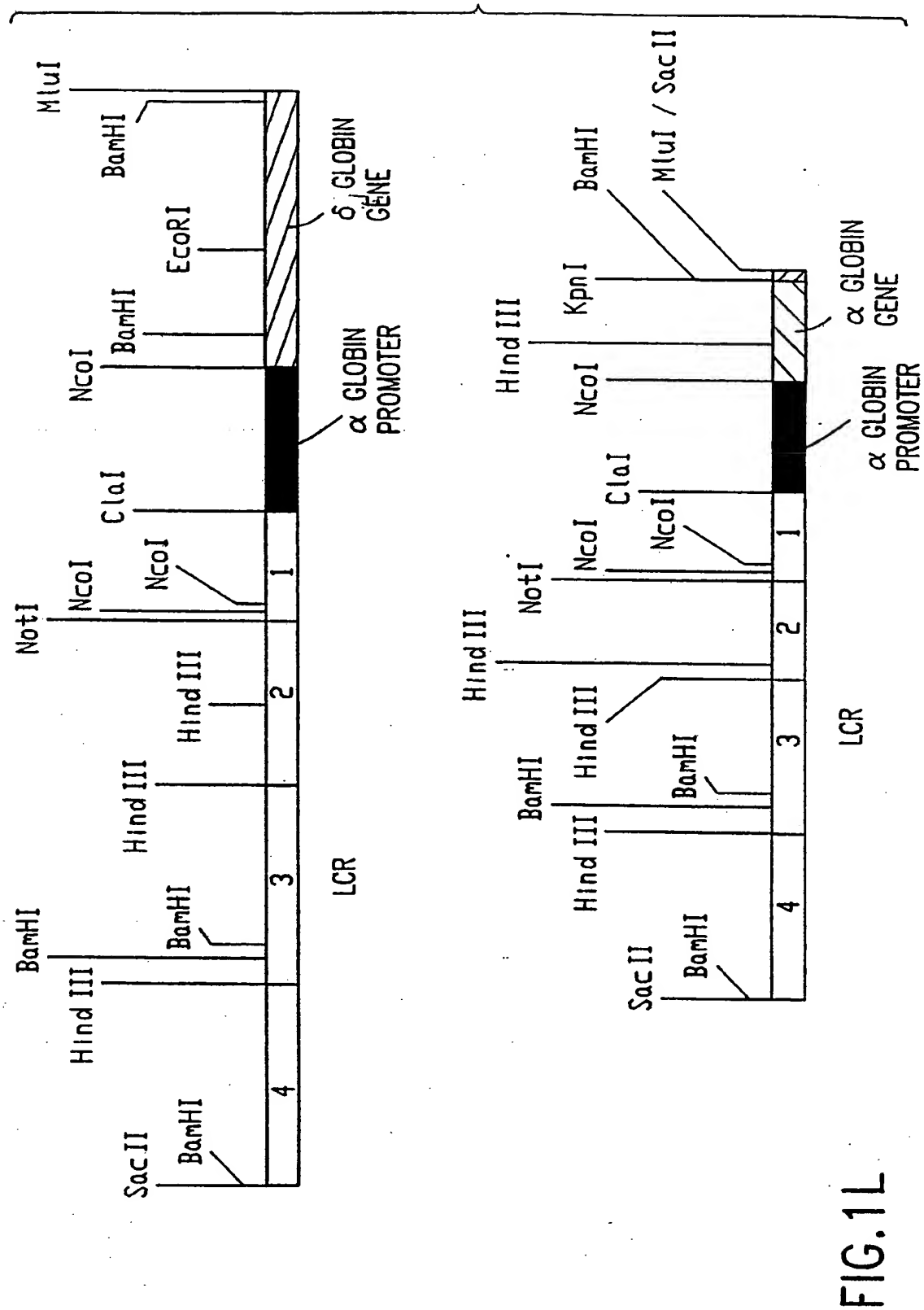
BamHI
EcoRI
BamHI
NcoI
KpnI
ClaI
Hind III
NcoI
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
SacII
δ-GLOBIN GENE
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
1
2
3
4
LCR

FIG. 1K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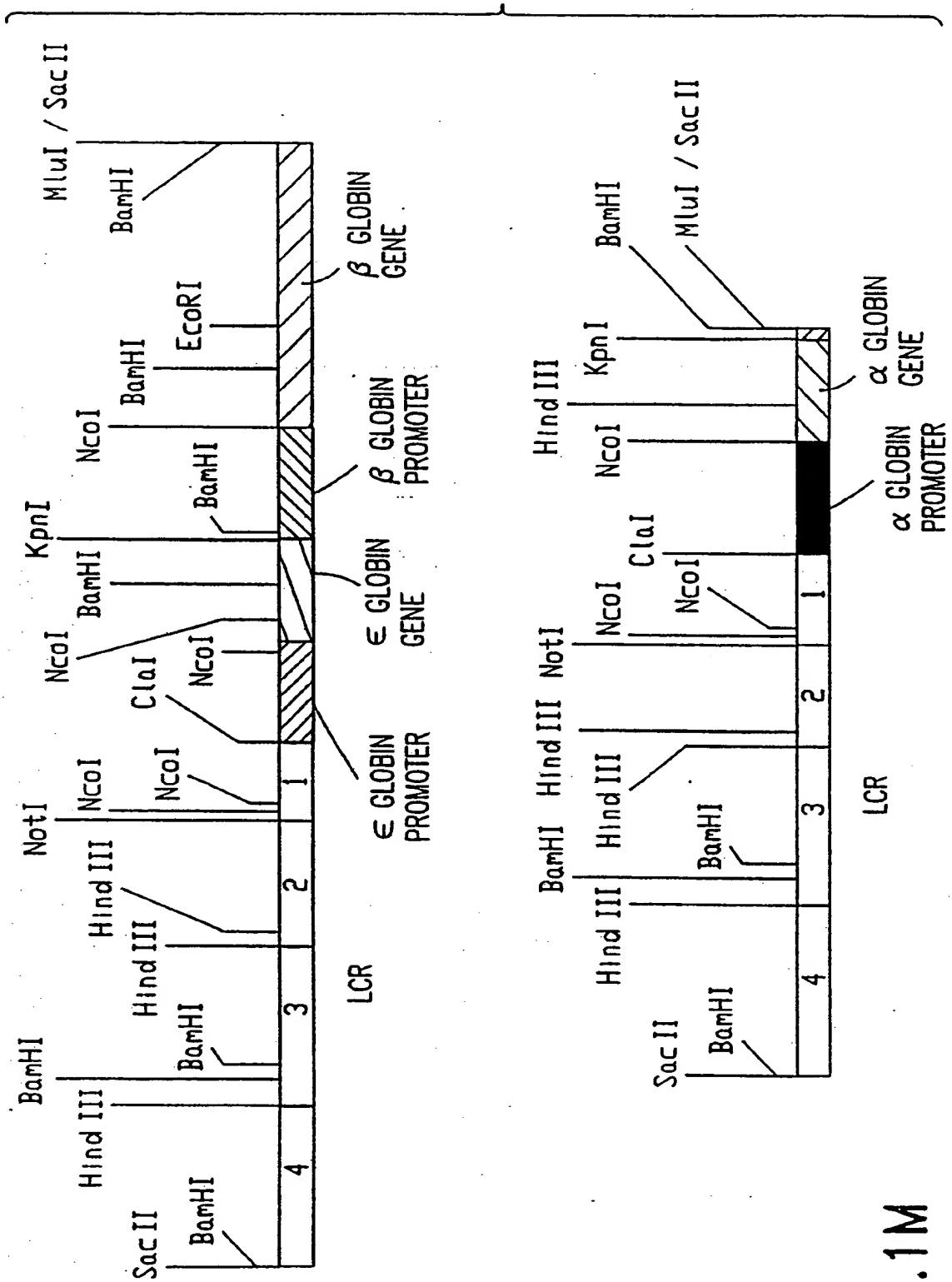
BamHI
δ GLOBIN GENE
EcoRI
BamHI
NcoI
α GLOBIN PROMOTER
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
SacII
BamHI
1
2
3
4
LCR
Hind III
Kpn I
BamHI
MluI / SacII
α GLOBIN GENE
NcoI
α GLOBIN PROMOTER
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
SacII
BamHI
1
2
3
4
LCR

FIG. 1L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BamHI
β GLOBIN GENE
EcoRI
BamHI
NcoI
β GLOBIN PROMOTER
BamHI
KpnI
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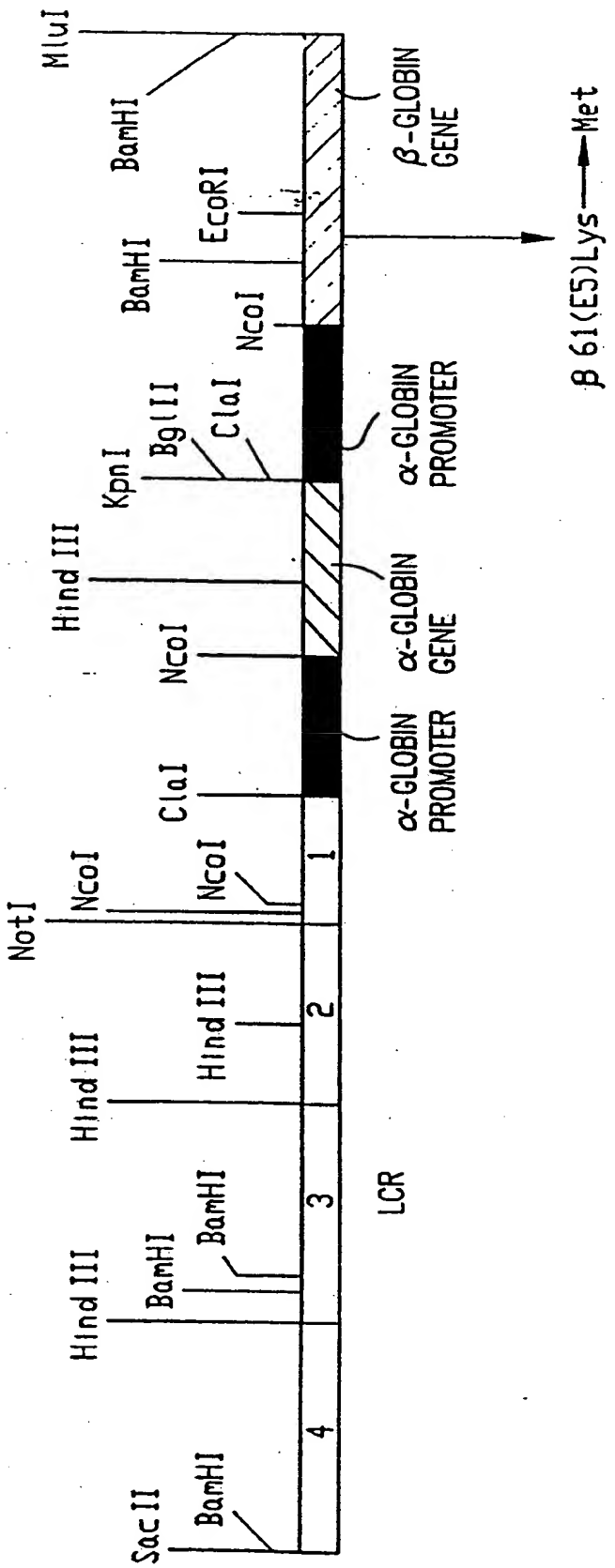
NcoI
ε GLOBIN GENE
NcoI
ClaI
NcoI
ε GLOBIN PROMOTER
NcoI
NotI
NcoI
HindIII
HindIII
BamHI
HindIII
BamHI
SacII
BamHI
1
2
3
4
LCR
HindIII
KpnI
BamHI
MluI / SacII
α GLOBIN GENE
NcoI
α GLOBIN PROMOTER
ClaI
NcoI
NcoI
NotI
HindIII
HindIII
BamHI
HindIII
BamHI
SacII
BamHI
1
2
3
4
LCR

FIG. 1M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BamHI
EcoRI
BamHI
NcoI
ClaI
BglII
KpnI
HindIII
NcoI
ClaI
NcoI
NcoI
NotI
HindIII
HindIII
BamHI
HindIII
BamHI
SacII
1
2
3
4
LCR
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
β-GLOBIN GENE
β 61(E5)Lys → Met

FIG.1N

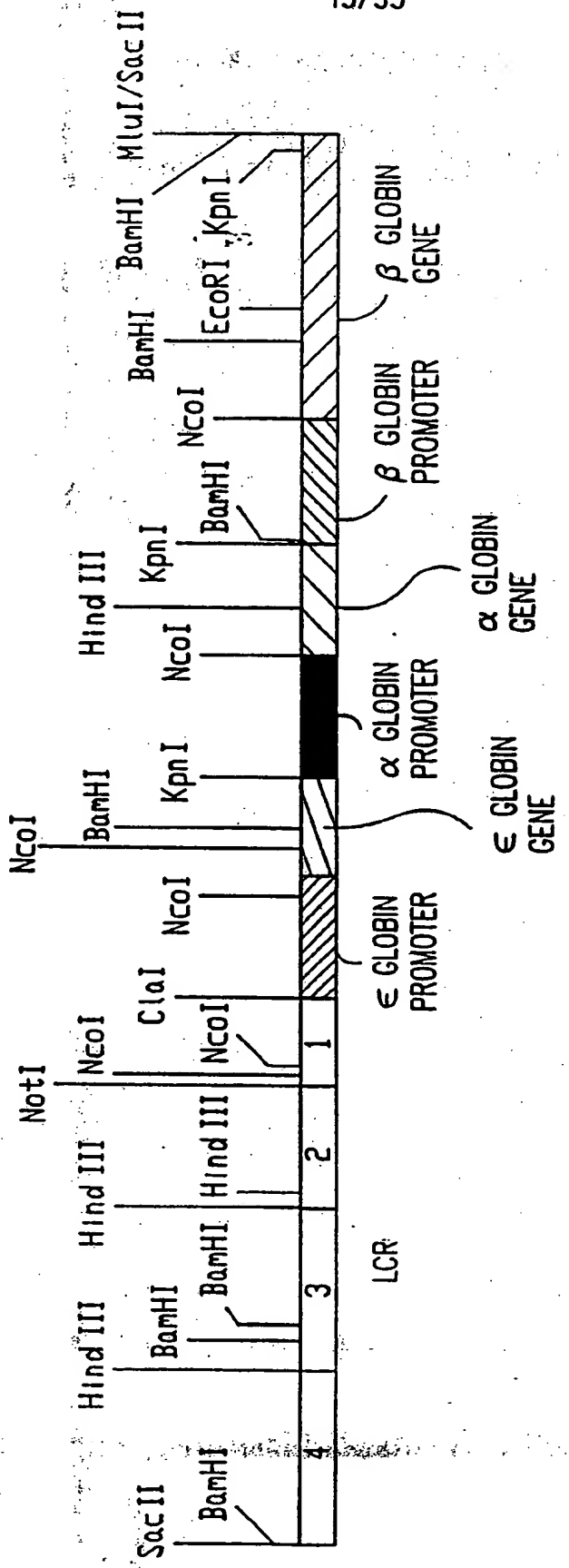

MluI/Sac II
BamHI
Kpn I
EcoRI
BamHI
NcoI
BamHI
Kpn I
Hind III
NcoI
BamHI
Kpn I
NcoI
NcoI
Cla I
NcoI
Not I
Hind III
BamHI
Hind III
Sac II
BamHI
β GLOBIN GENE
β GLOBIN PROMOTER
α GLOBIN GENE
α GLOBIN PROMOTER
ε GLOBIN GENE
ε GLOBIN PROMOTER
1
2
3
4
LCR

FIG.10

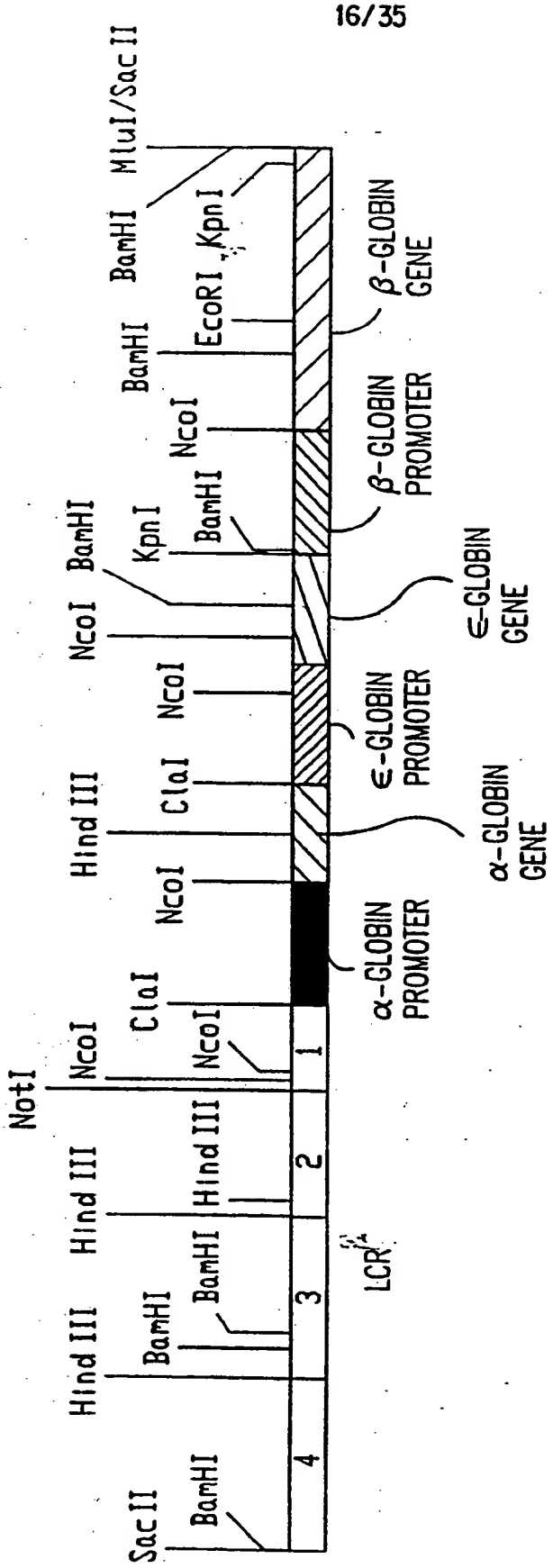

MluI/Sac II
BamHI
KpnI
EcoRI
BamHI
NcoI
BamHI
KpnI
NcoI
NcoI
ClaI
Hind III
NcoI
ClaI
NcoI
NotI
Hind III
BamHI
Hind III
BamHI
SacII
1
2
3
4
LCR
β-GLOBIN GENE
β-GLOBIN PROMOTER
∈-GLOBIN GENE
∈-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER

FIG.1P

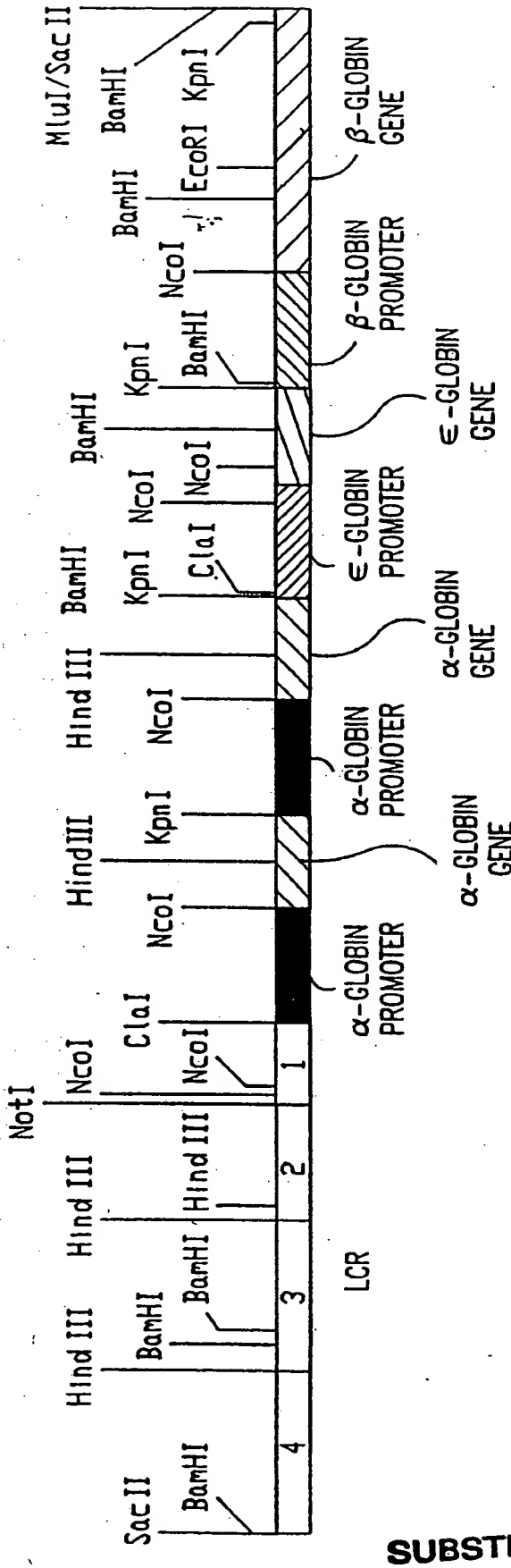

MluI/SacII
BamHI
KpnI
EcoRI
BamHI
NcoI
BamHI
KpnI
NcoI
BamHI
KpnI
ClaI
NcoI
HindIII
NcoI
HindIII
KpnI
NcoI
ClaI
NcoI
NotI
HindIII
BamHI
HindIII
BamHI
SacII
BamHI
β-GLOBIN GENE
β-GLOBIN PROMOTER
ε-GLOBIN GENE
ε-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
LCR
1
2
3
4

FIG.1Q

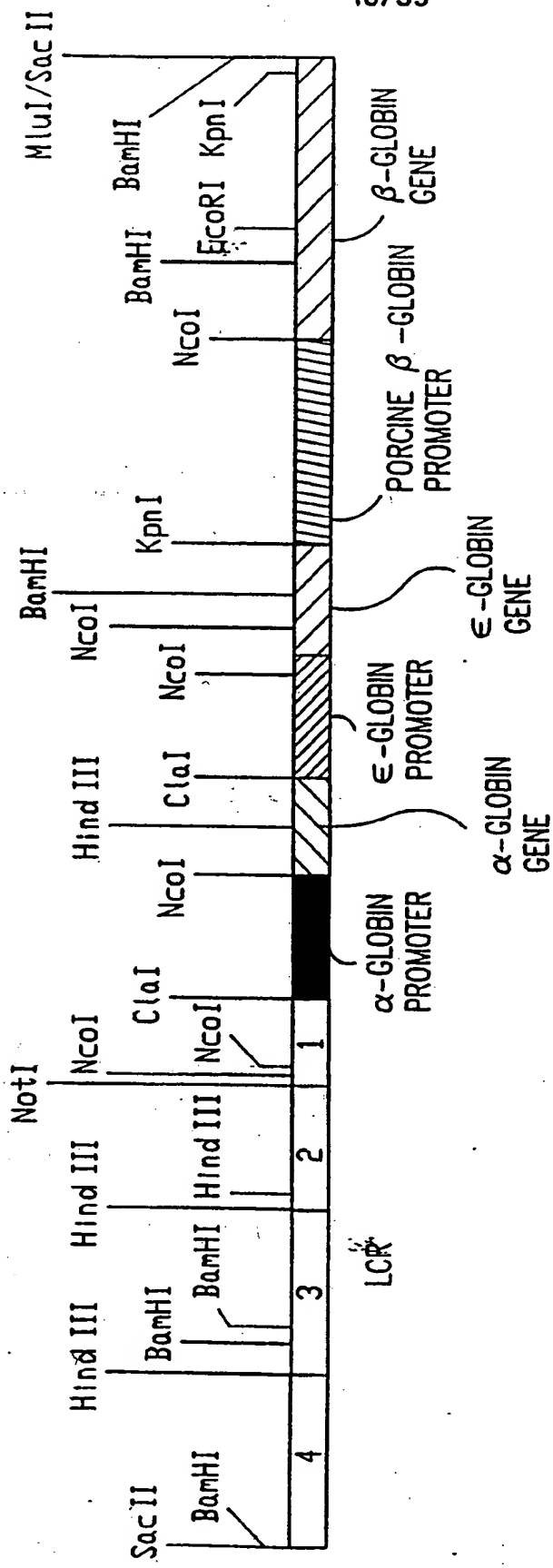

MluI/SacII
BamHI
KpnI
EcoRI
BamHI
NcoI
PORCINE β-GLOBIN PROMOTER
β-GLOBIN GENE
KpnI
BamHI
NcoI
NcoI
ε-GLOBIN PROMOTER
ε-GLOBIN GENE
HindIII
ClaI
NcoI
α-GLOBIN PROMOTER
α-GLOBIN GENE
NcoI
ClaI
NcoI
NotI
HindIII
HindIII
BamHI
BamHI
HindIII
SacII
BamHI
1
2
3
4
LCR

FIG.1R

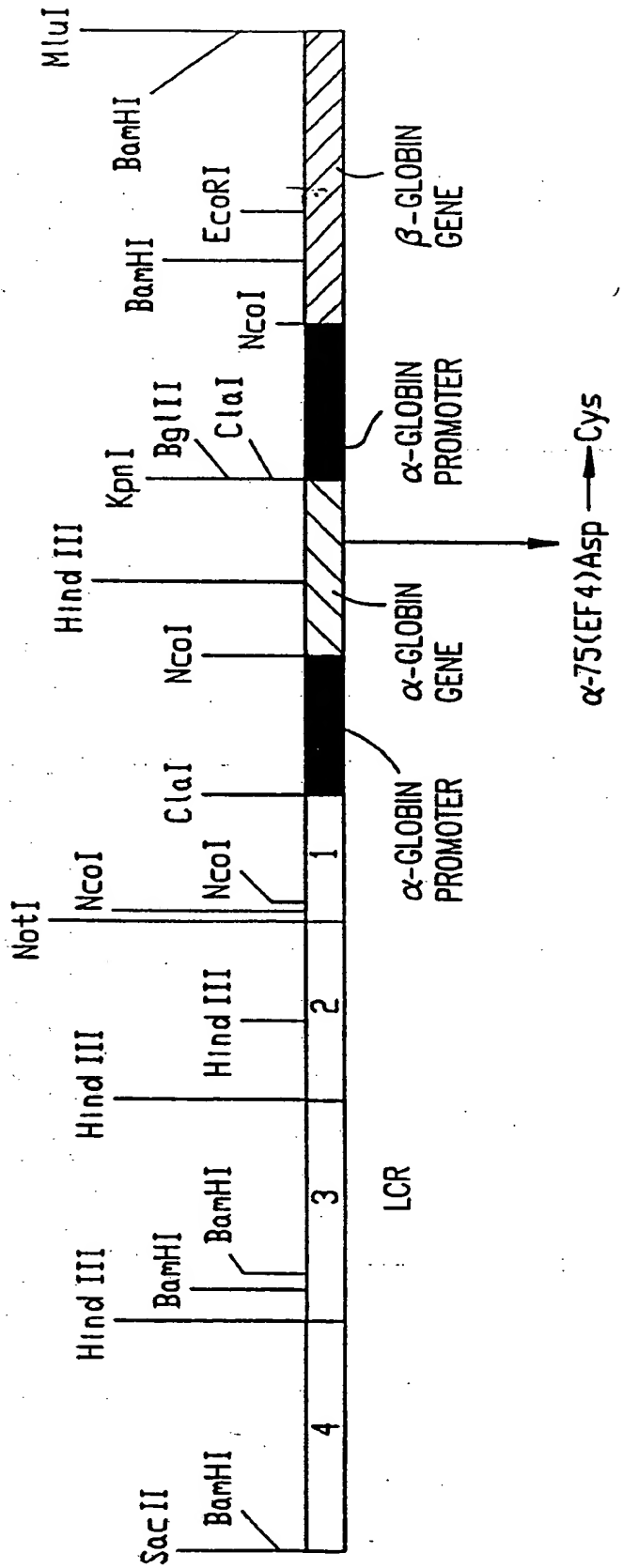

MluI
BamHI
EcoRI
BamHI
NcoI
KpnI
BglII
ClaI
Hind III
NcoI
ClaI
NcoI
NotI
NcoI
Hind III
Hind III
BamHI
BamHI
Hind III
BamHI
SacII
1
2
3
4
LCR
β-GLOBIN GENE
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
α-75(EF4)Asp → Cys

FIG.1S

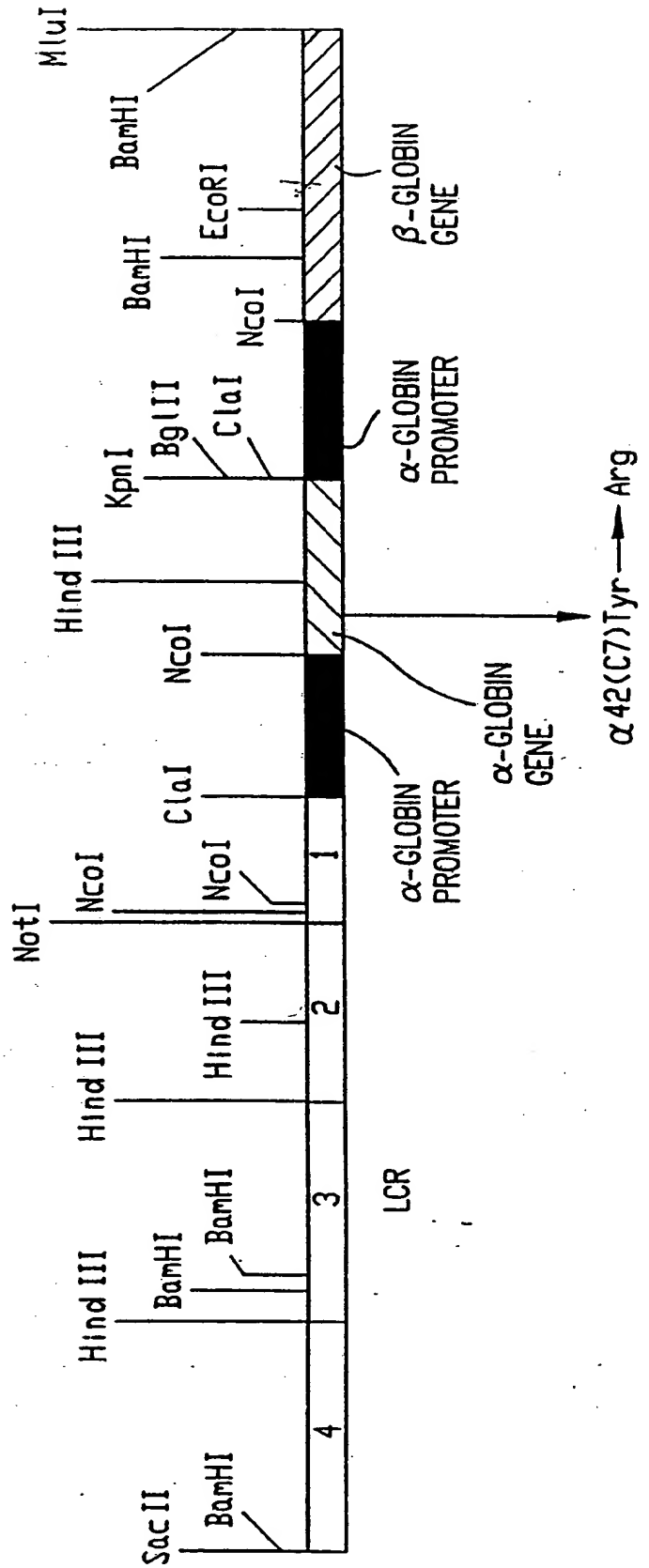

MluI
BamHI
EcoRI
BamHI
NcoI
ClaI
BglII
KpnI
Hind III
NcoI
ClaI
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
BamHI
SacII
1
2
3
4
LCR
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
β-GLOBIN GENE
α42(C7)Tyr → Arg

FIG. 1T

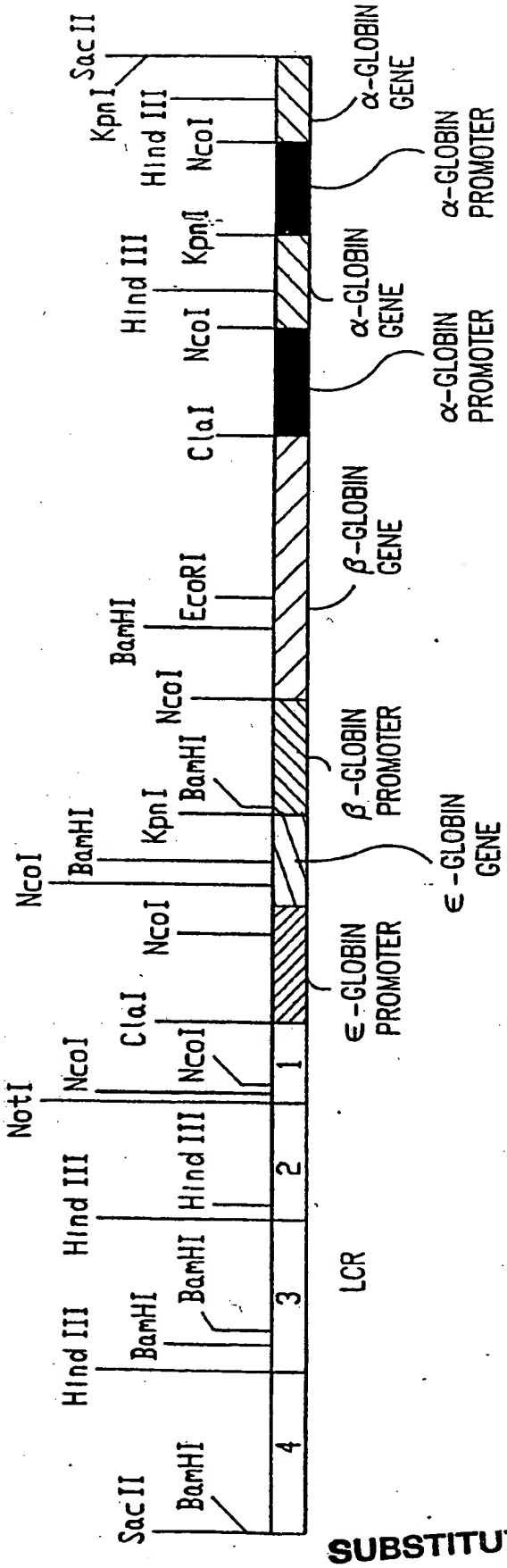

SacII
BamHI
Hind III
BamHI
Hind III
BamHI
Hind III
NotI
NcoI
ClaI
NcoI
NcoI
BamHI
KpnI
BamHI
NcoI
BamHI
EcoRI
ClaI
NcoI
Hind III
KpnI
NcoI
Hind III
KpnI
NcoI
SacII
1
2
3
4
LCR
ε-GLOBIN PROMOTER
ε-GLOBIN GENE
β-GLOBIN PROMOTER
β-GLOBIN GENE
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
α-GLOBIN GENE

FIG. 1U

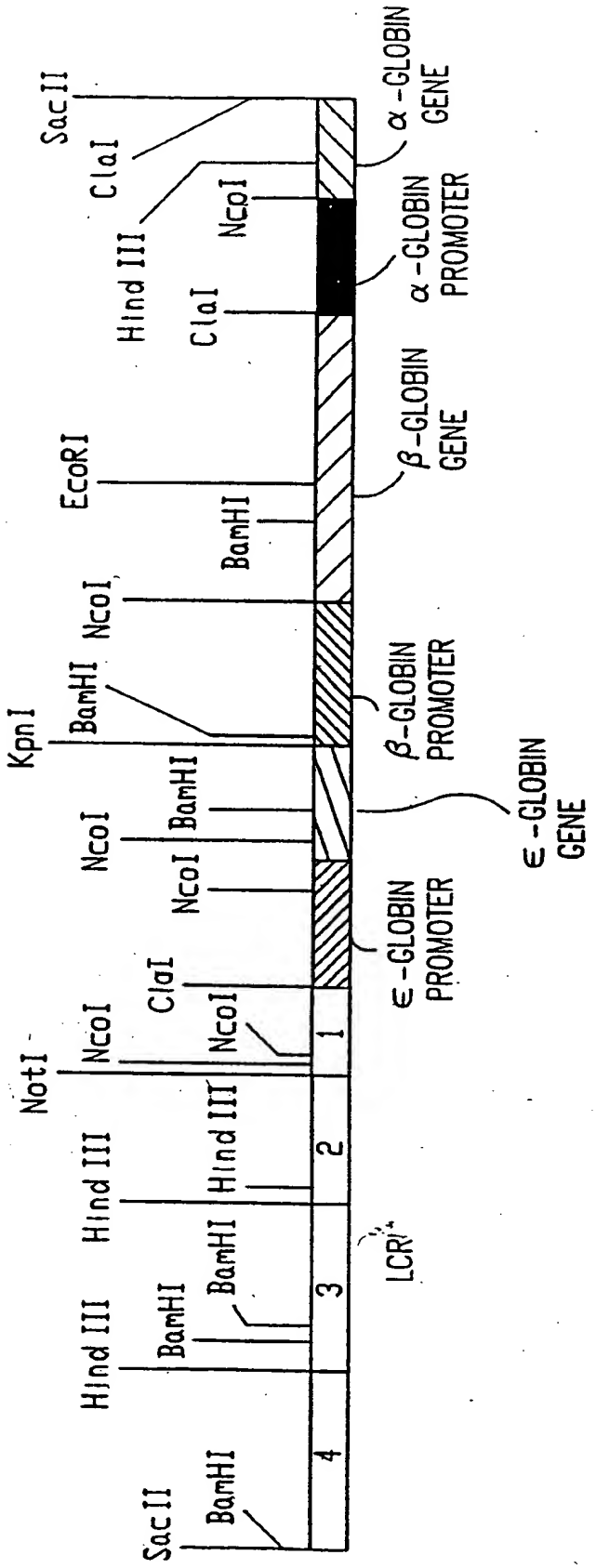

SacII
ClaI
Hind III
NcoI
ClaI
EcoRI
BamHI
NcoI
KpnI
BamHI
NcoI
BamHI
NcoI
NcoI
ClaI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
SacII
BamHI
1
2
3
4
LCR
α-GLOBIN GENE
α-GLOBIN PROMOTER
β-GLOBIN GENE
β-GLOBIN PROMOTER
ε-GLOBIN GENE
ε-GLOBIN PROMOTER

FIG. 1V

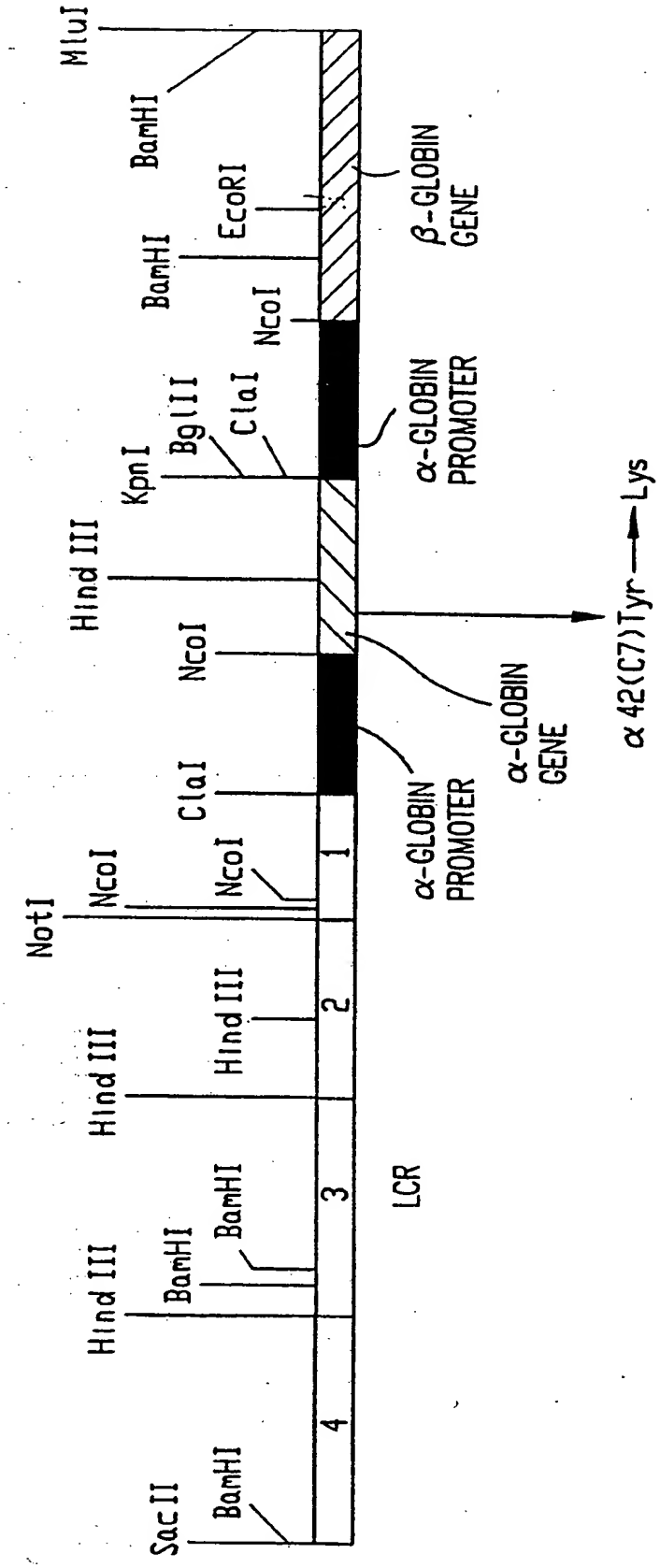

MluI
BamHI
EcoRI
BamHI
NcoI
KpnI
BglII
ClaI
HindIII
NcoI
ClaI
NcoI
NotI
NcoI
HindIII
HindIII
BamHI
BamHI
HindIII
SacII
BamHI
1
2
3
4
LCR
α-GLOBIN PROMOTER
α-GLOBIN GENE
α-GLOBIN PROMOTER
β-GLOBIN GENE
α 42(C7) Tyr → Lys

FIG. 1W

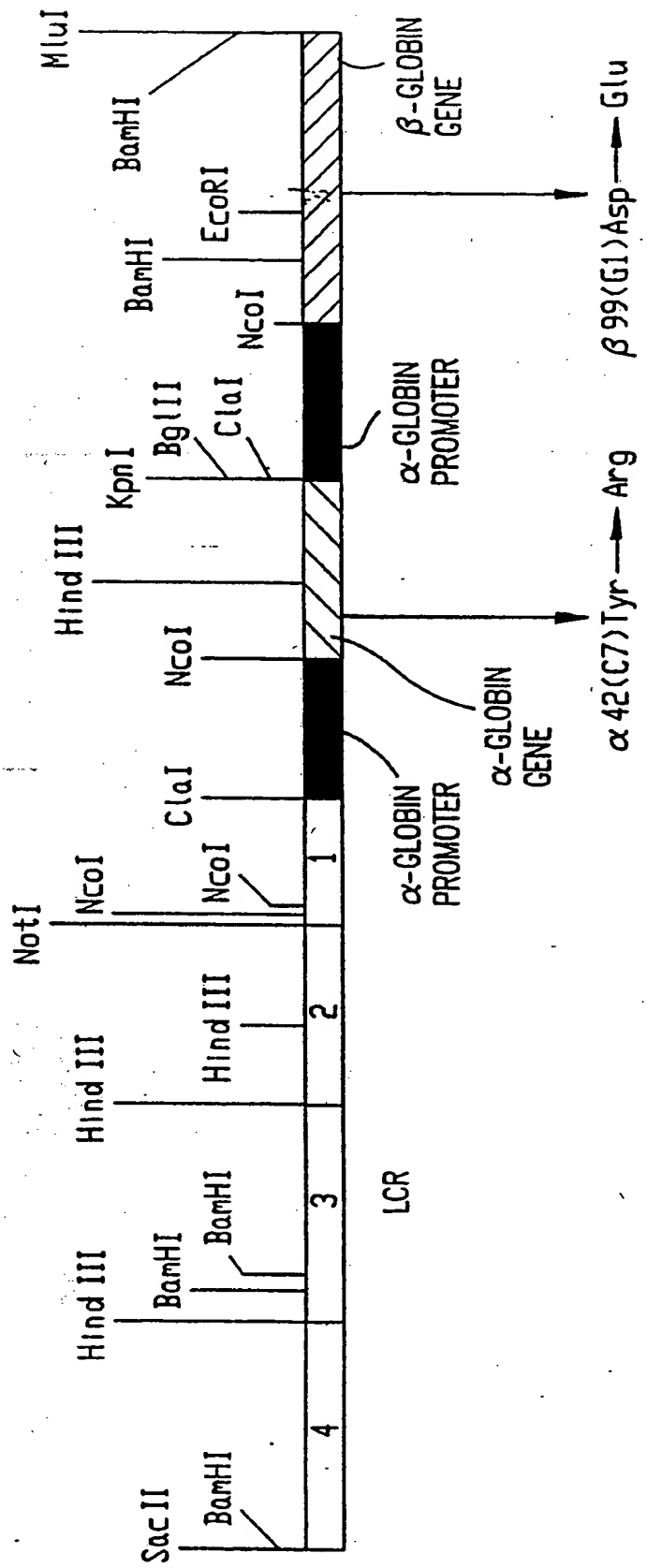

MluI
BamHI
β-GLOBIN GENE
EcoRI
BamHI
NcoI
α-GLOBIN PROMOTER
KpnI
Bg1II
ClaI
Hind III
NcoI
α-GLOBIN GENE
ClaI
α-GLOBIN PROMOTER
NcoI
NcoI
NotI
Hind III
Hind III
BamHI
BamHI
Hind III
LCR
SacII
BamHI
1
2
3
4
β99(G1)Asp → Glu
α42(C7)Tyr → Arg

FIG. 1X

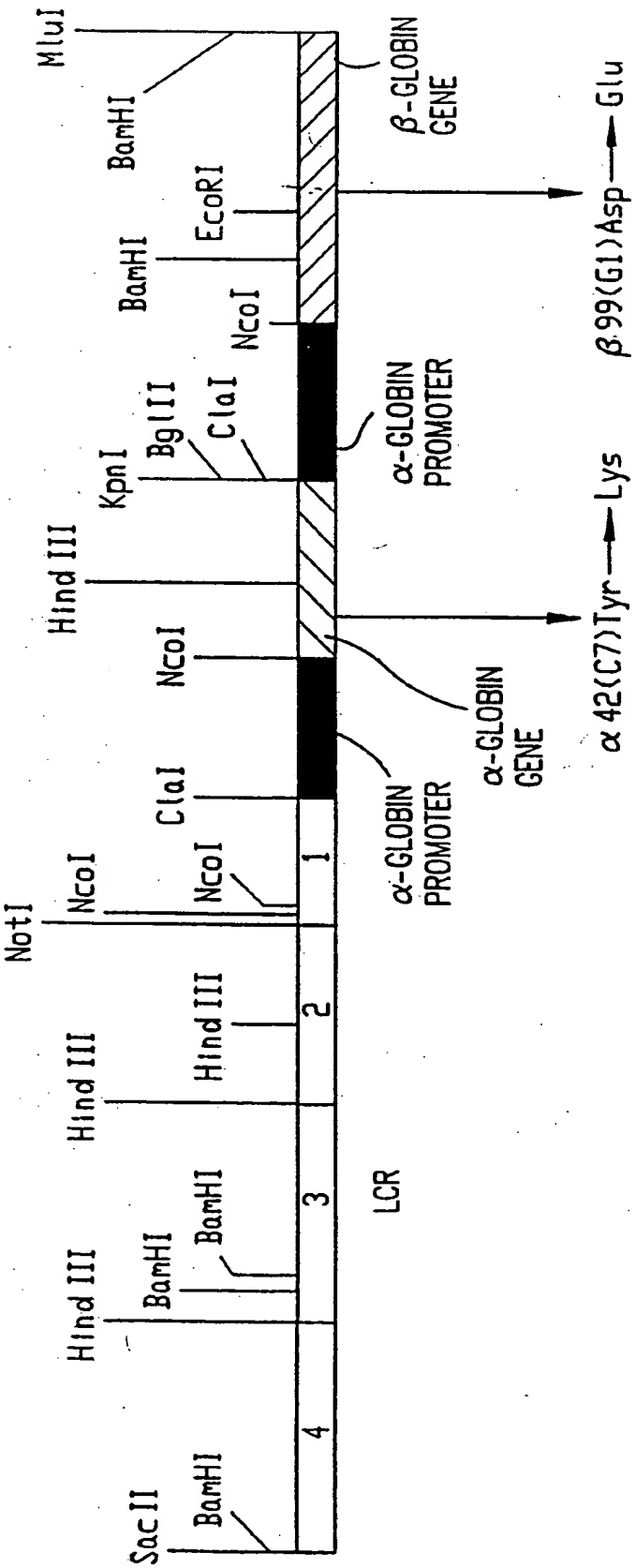

MluI
BamHI
β-GLOBIN GENE
EcoRI
BamHI
NcoI
α-GLOBIN PROMOTER
β 99(G1)Asp
Glu
Bg1II
ClaI
KpnI
Hind III
NcoI
α-GLOBIN GENE
α 42(C7)Tyr
Lys
ClaI
NcoI
NcoI
α-GLOBIN PROMOTER
NotI
NcoI
1
Hind III
Hind III
2
BamHI
Hind III
BamHI
3
LCR
4
SacII
BamHI

FIG. 1Y

FIG. 2

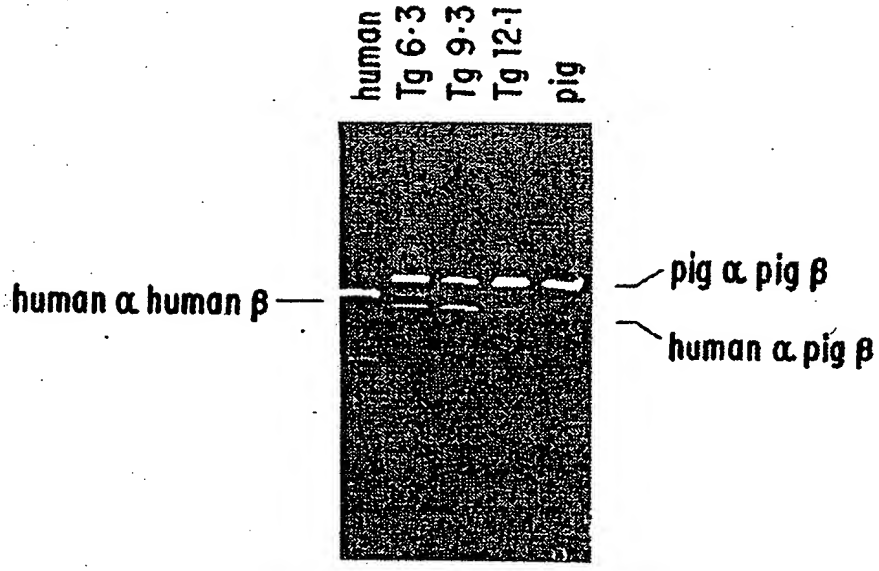

human
Tg 6·3
Tg 9·3
Tg 12·1
pig
human α human β
pig α pig β
human α pig β

FIG. 3A

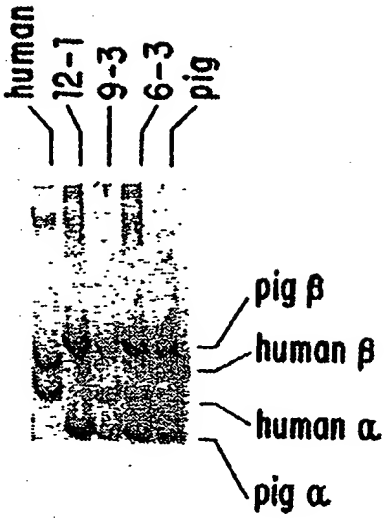

human
12-1
9-3
6-3
pig
pig β
human β
human α
pig α

FIG. 3B

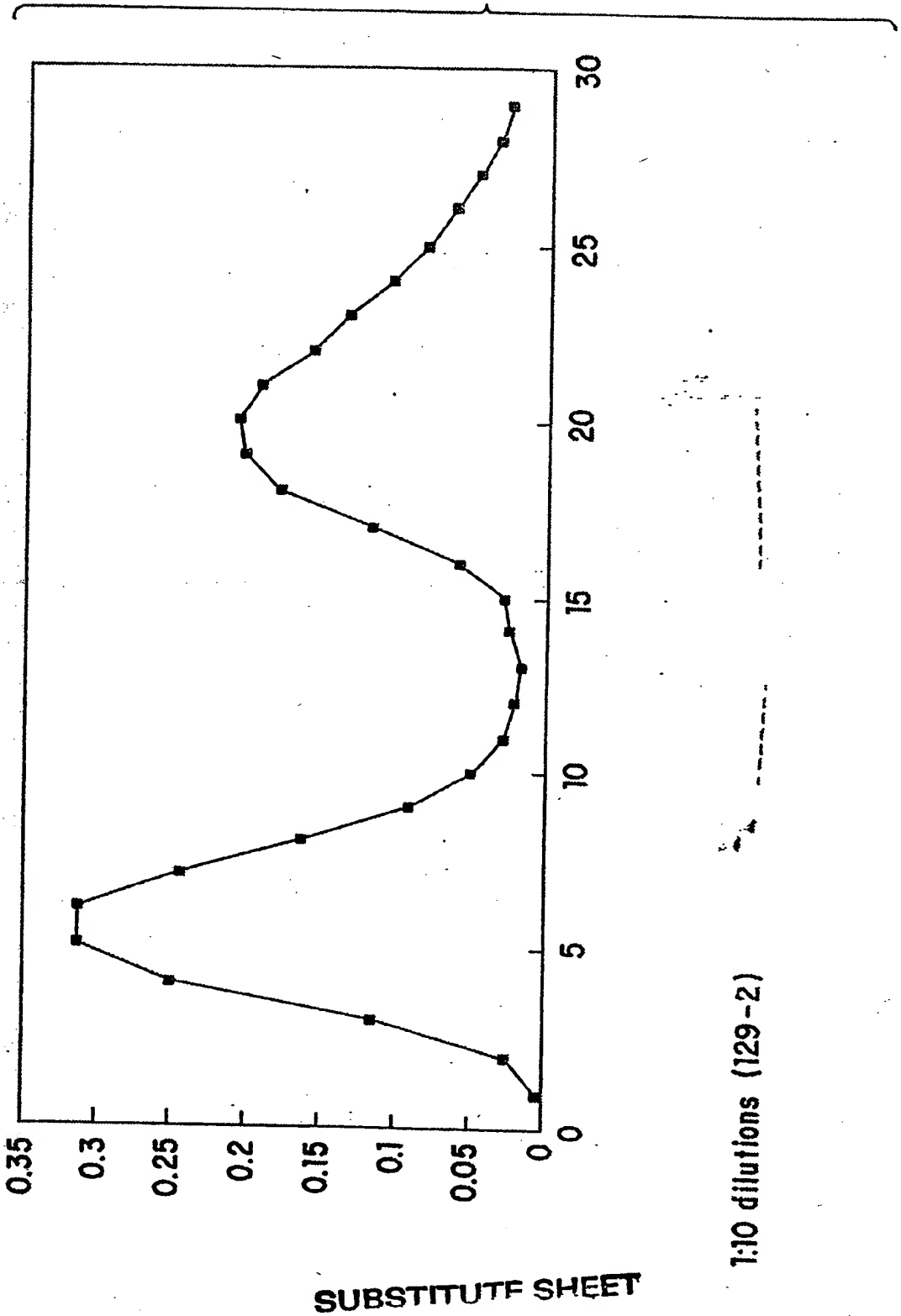
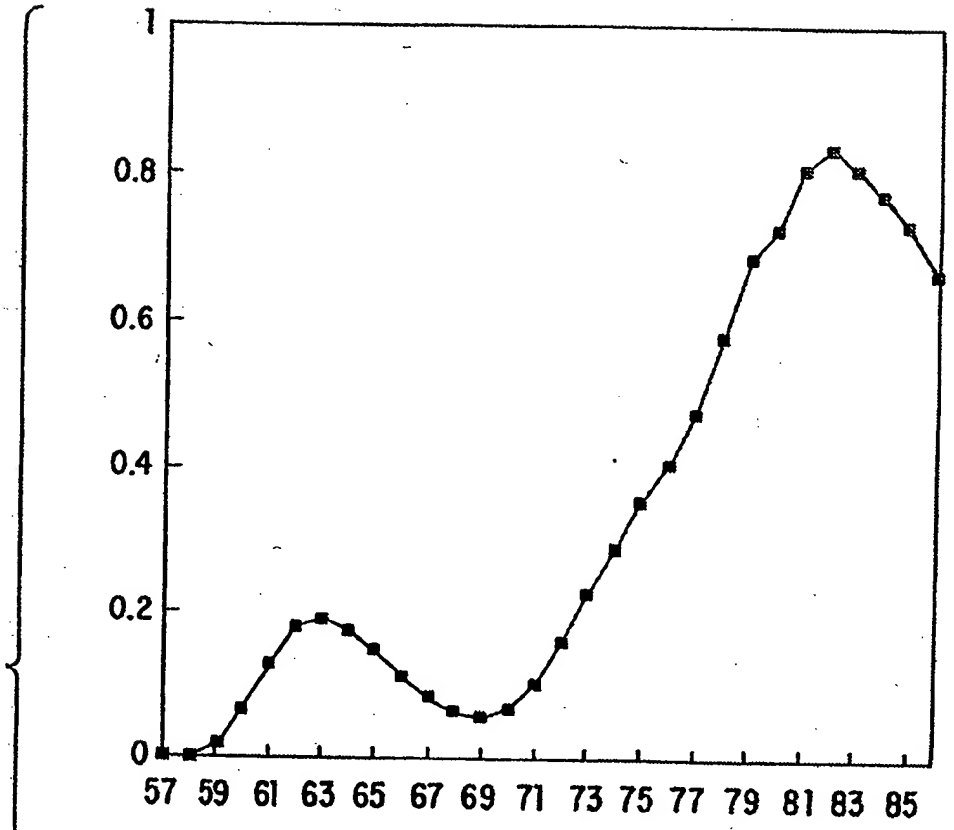


FIG. 4A


1
0.8
0.6
0.4
0.2
0
57 59 61 63 65 67 69 71 73 75 77 79 81 83 85

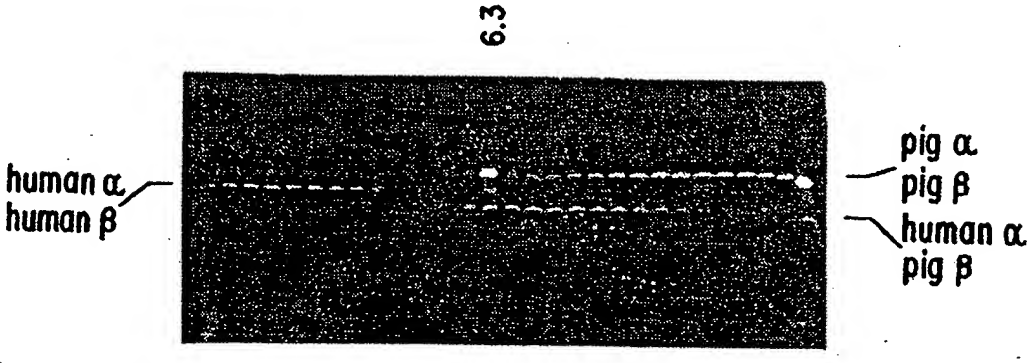

6.3
human α
human β
pig α
pig β
human α
pig β

FIG. 4B

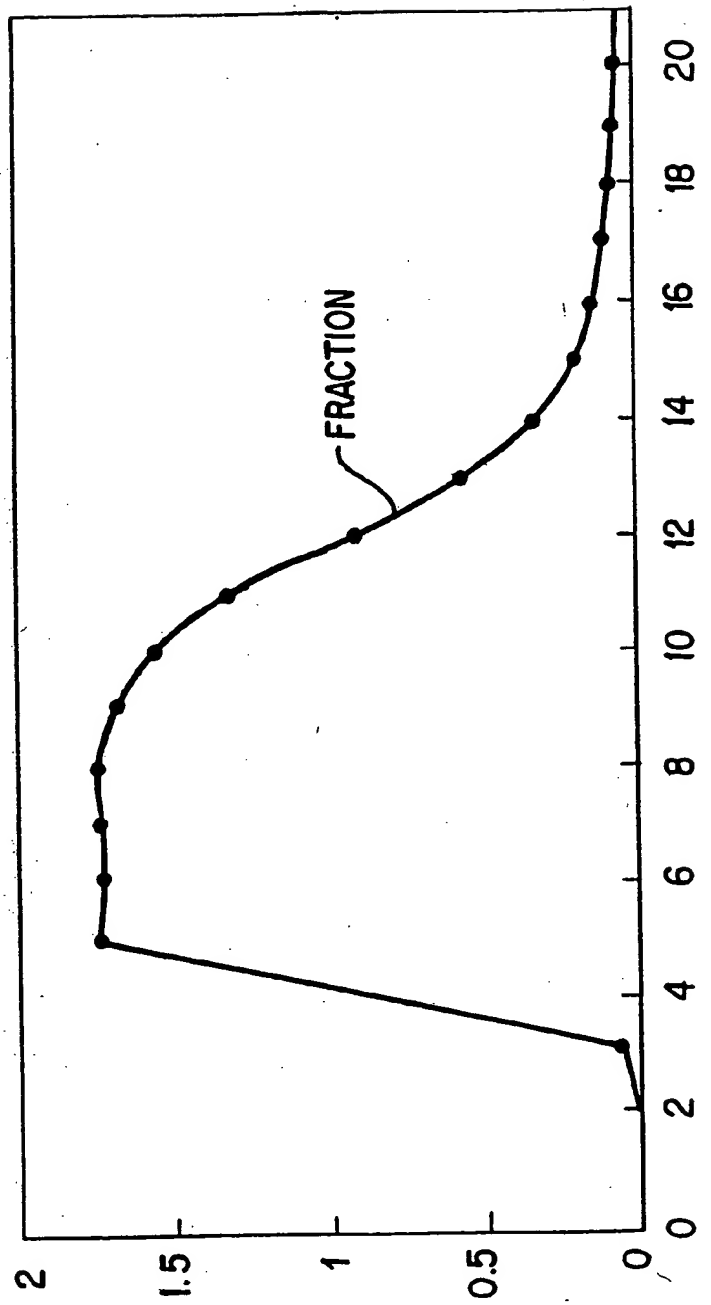


FIG. 4C

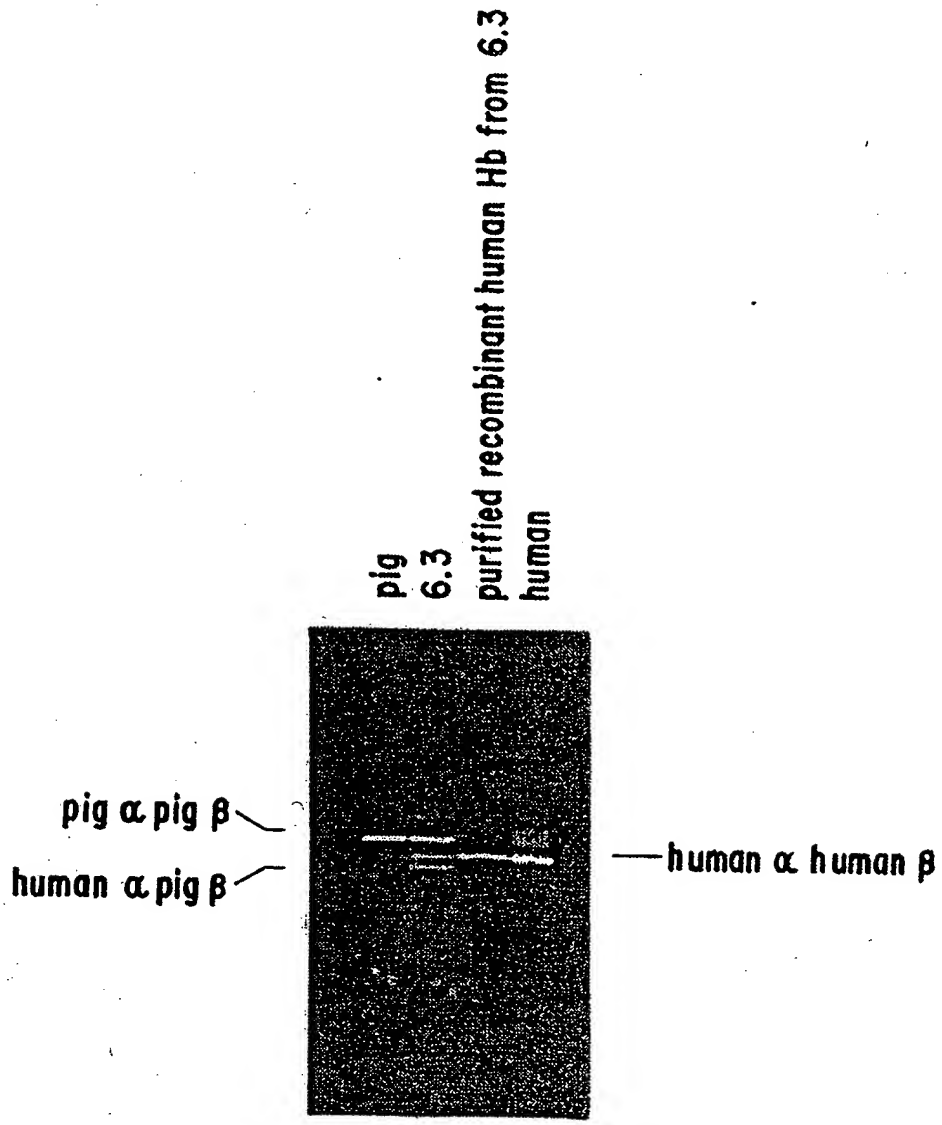

pig
6.3
purified recombinant human Hb from 6.3
human
pig α pig β
human α pig β
human α human β

FIG. 4D

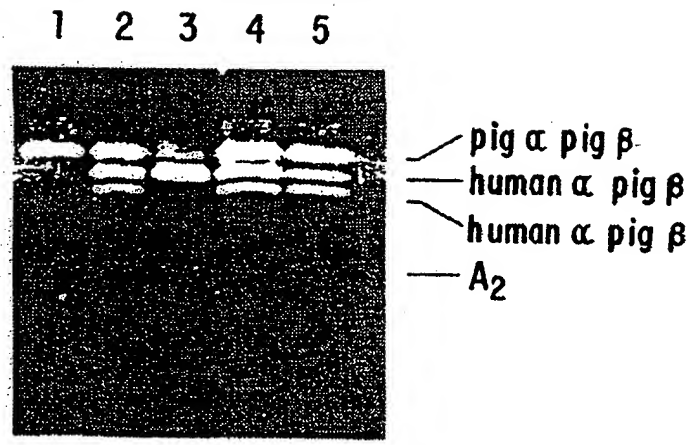


FIG. 5

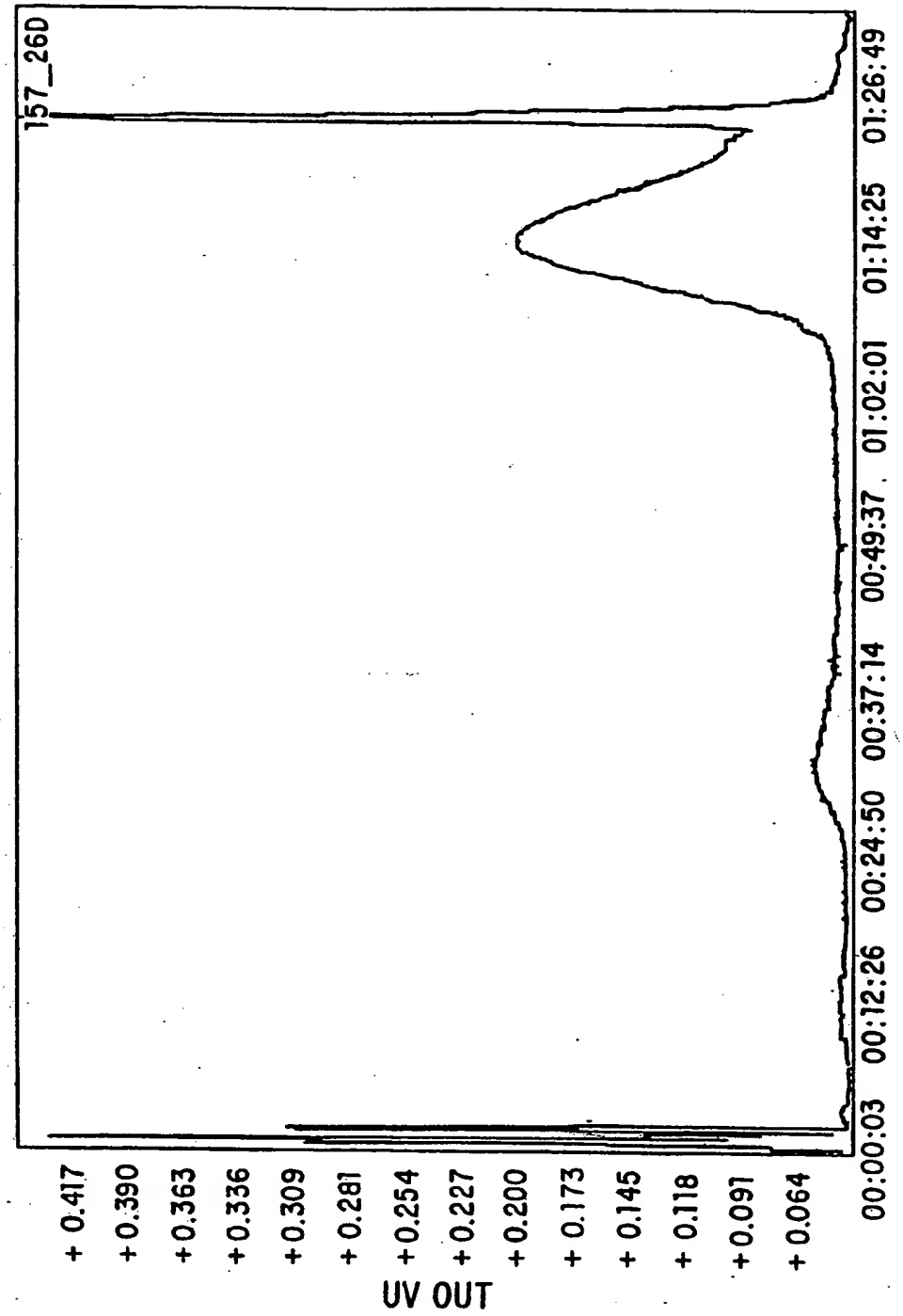

157_26D
+ 0.417
+ 0.390
+ 0.363
+ 0.336
+ 0.309
+ 0.281
+ 0.254
+ 0.227
+ 0.200
+ 0.173
+ 0.145
+ 0.118
+ 0.091
+ 0.064
UV OUT
00:00:03
00:12:26
00:24:50
00:37:14
00:49:37
01:02:01
01:14:25
01:26:49
TIME - hh:mm:ss

FIG. 6

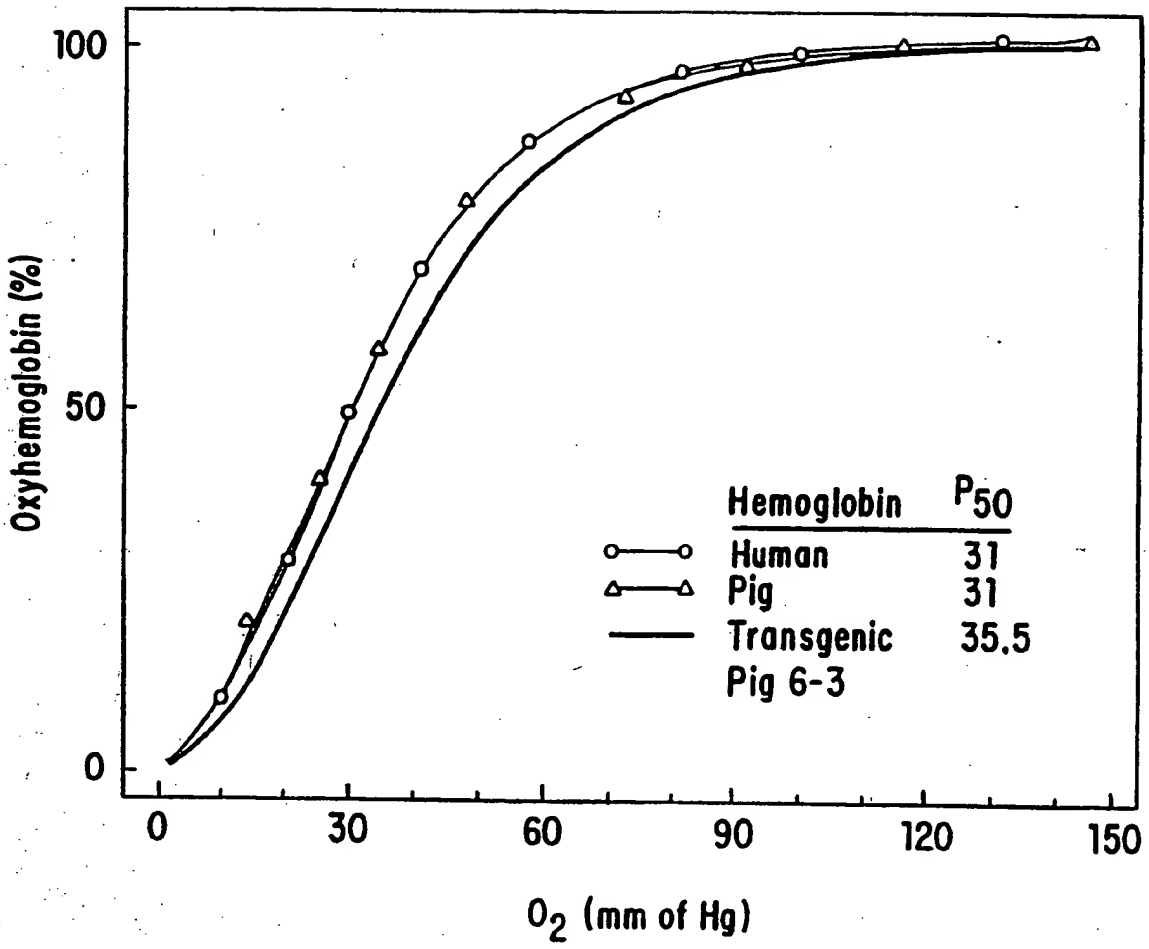

100
50
0
Oxyhemoglobin (%)
0
30
60
90
120
150
O_2 (mm of Hg)
Hemoglobin
P_{50}
Human
31
Pig
31
Transgenic Pig 6-3
35.5

FIG. 7A

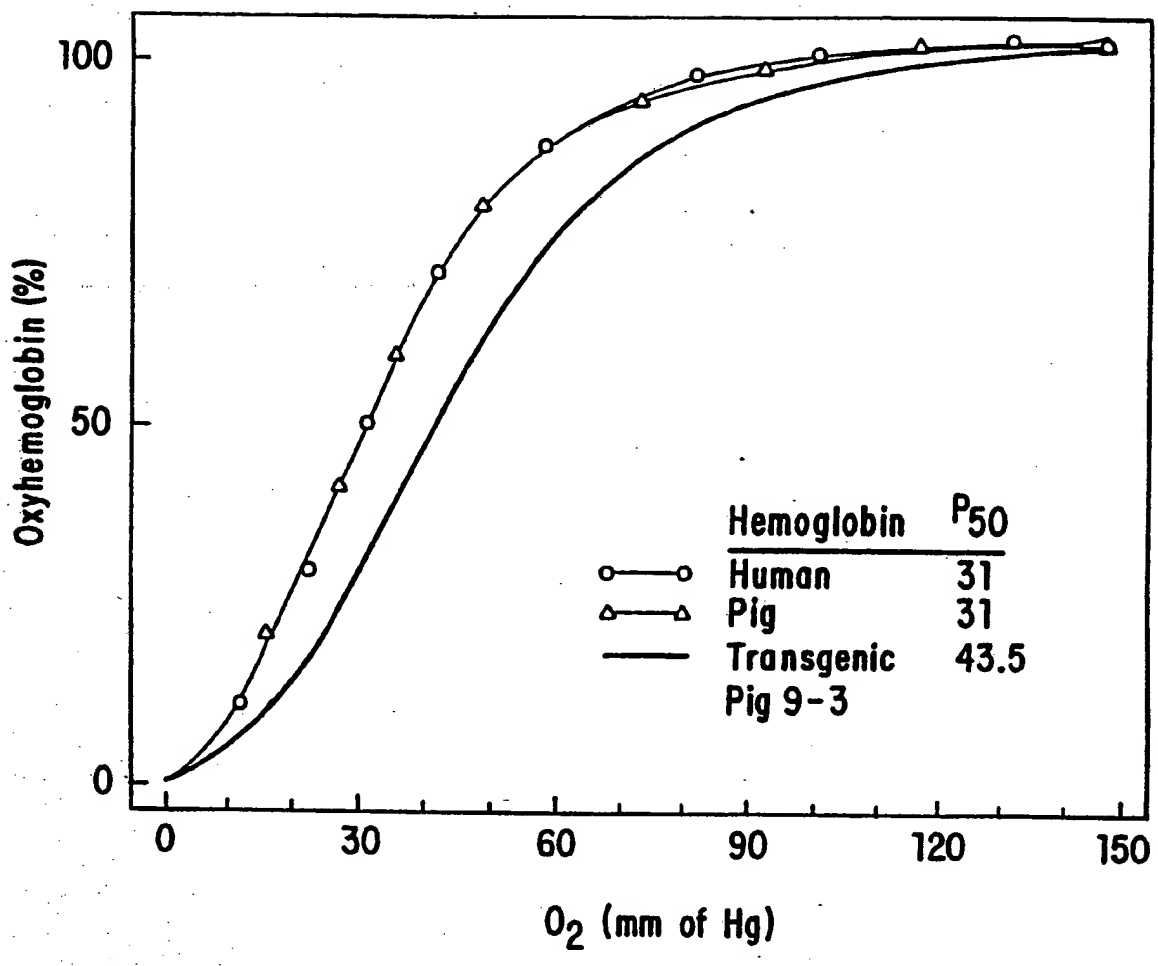

100
50
0
Oxyhemoglobin (%)
0
30
60
90
120
150
O_2 (mm of Hg)
Hemoglobin P50
Human 31
Pig 31
Transgenic Pig 9-3 43.5

FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05000

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00; C12P 21/06; C07K 3/00, 7/00, 13/00
US CL :800/2; 435/69.6; 530/416; 935/9, 60
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/69.6; 530/416; 935/9, 60

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Science, volume 245, issued 01 September 1989, R.S. Behringer et al, "Synthesis of Functional Human Hemoglobin in Transgenc Mice", pages 971-973, entire document.	1-38
X	Nature, Volume 315, issued 20 June 1985, R.E. Hammer et al, "Production of Transgenic Rabbits, Sheep and Pigs by Microinjection", pages 680-683, entire document.	1-38
X	Proceedings of the National Academy of Science, Volume 81, issued September 1984, K. Young et al, "Stable Transfer and Expression of Exogenous Human Globin Genes in Human Erythroleukemia (K562) Cells", 5315-5319, entire document.	1-38
X	Nucleic Acids Research, Volume 76, issued 1981, A. Riggs, "Preparation of Blood Hemoglobins of Vertebrates", pages 5-29, Methods in Enzymology, entire document.	34-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 August 1992	20 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DEBORAH CROUCH, PH.D. Telephone No. (703) 308-1126

Form PCT/ISA/210 (second sheet)(July 1992)*

Appendix E

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A01K 67/00, 67/027, C12N 15/90 C12P 21/06

A1

(11) International Publication Number: WO 93/25071

(43) International Publication Date: 23 December 1993 (23.12.93)

(21) International Application Number: PCT/US93/05629

(22) International Filing Date: 11 June 1993 (11.06.93)

(30) Priority data:

897,648	12 June 1992 (12.06.92)	US
987,890	8 December 1992 (08.12.92)	US
030,897	15 March 1993 (15.03.93)	US

(71) Applicant: DNX CORPORATION [US/US]; 303B College Road East, Princeton Forrestal Area, Princeton, NJ 08540 (US).

(72) Inventors: LOGAN, John, S. ; 347 B Old York Road, Robbinsville, NJ 08691 (US). HOLTZMAN, Steven, H. ; 15 Sherbrooke Drive, Princeton Junction, NJ 08550 (US). O'DONNELL, J., Kevin ; 2822 Hearth Place, Doylestown, PA 18901 (US). PILDER, Stephen, H. ; 63-10 Raven's Crest Drive, Plainsboro, NJ 08536 (US). PINKERT, Carl, A. ; 1998 Lakemont Drive, Bessemer, AL 35023 (US). SWANSON, Mark, E. ; 16 Hereford Drive, Princeton Junction, NJ 08550 (US). KELLER, Hilary ; 3613 Quail Ridge Drive, Plainsboro, NJ 08536 (US). SHARMA, Ajay ; 24 Feiler Court, Lawrenceville, NJ 08648 (US). PARSONS, Cynthia, T. ; 26 Groendyke Lane, Plainsboro, NJ 08536 (US). KUMAR, Ramesh ; 60 Yard Road, Pennington, NJ 08534 (US). WHITE, Steven, P. ; 566 Edison Drive, Highstown, NJ 08520 (US).

(74) Agents: MISROCK, S., Leslie et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York; NY 10036 (US).

(81) Designated States: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS

(57) Abstract

The present invention relates to the use of transgenic pigs for the production of human hemoglobin in which, in certain embodiments, the pig beta globin promoter is used to facilitate the expression of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.

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BE	Belgium	GN	Guinea	NO	Norway
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BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
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DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS

1. INTRODUCTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.

2. BACKGROUND OF THE INVENTION

2.1. HEMOGLOBIN

Oxygen absorbed through the lungs is carried by hemoglobin in red blood cells for delivery to tissues throughout the body. At high oxygen tensions, such as those found in the proximity of the lungs, oxygen binds to hemoglobin, but is released in areas of low oxygen tension, where it is needed.

Each hemoglobin molecule consists of two alpha globin and two beta globin subunits. Each subunit, in turn, is noncovalently associated with an iron-containing heme group capable of carrying an oxygen molecule. Thus, each hemoglobin tetramer is capable of binding four molecules of oxygen. The subunits work together in switching between two conformational states to facilitate uptake and release of oxygen at the lungs and tissues, respectively. This effect is commonly referred to as heme-heme interaction or cooperativity.

The hemoglobins of many animals are able to interact with biologic effector molecules that can further enhance oxygen binding and release. This enhancement is manifested in changes which affect the allosteric equilibrium between the two conformational states of hemoglobin. For example, human and pig hemoglobin can bind 2, 3 diphosphoglycerate (2,3 DPG),

which influences the equilibrium between the two conformational states of the tetramer and has the net effect of lowering the overall affinity for oxygen at the tissue level. As a result, 2,3-DPG increases the efficiency of oxygen delivery to the tissues.

2.2. GLOBIN GENE EXPRESSION

Hemoglobin protein is expressed in a tissue specific manner in red blood cells where it accounts for approximately ninety percent of total cellular protein. Thus, red blood cells, which have lost their nucleus and all but a minimal number of organelles, are effectively membrane-enclosed packets of hemoglobin dedicated to oxygen transfer.

Humans and various other species produce different types of hemoglobin during embryonic, fetal, and adult developmental periods. Therefore, the factors that influence globin gene expression must be able to achieve tissue specific control, quantitative control, and developmentally regulated control of globin expression.

Human globin genes are found in clusters on chromosome 16 for alpha (α) globin and chromosome 11 for beta (β) globin. The human beta globin gene cluster consists of about 50 kb of DNA that includes one embryonic gene encoding epsilon (ϵ) globin, two fetal genes encoding gamma (γ) G and gamma A globin, and two adult genes encoding delta (δ) and beta (β) globin, in that order (Fritsch et al., 1980, Cell 19:959-972).

It has been found that DNA sequences both upstream and downstream of the β globin translation initiation site are involved in the regulation of β globin gene expression (Wright et al., 1984, Cell 38:263). In particular, a series of four Dnase I super hypersensitive sites (now referred to as the locus control region, or LCR) located about 50

kilobases upstream of the human beta globin gene are extremely important in eliciting properly regulated beta globin locus expression (Tuan et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 83:1359-1363; PCT Patent Application WO 8901517 by Grosveld; Behringer et al., 1989, Science 245:971-973; Enver et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037; Hanscombe et al., 1989, Genes Dev. 3:1572-1581; Van Assendelft et al., 1989, Cell 56:967-977; Grosveld et al., 1987, Cell 51:975-985).

2.3 THE NEED FOR A BLOOD SUBSTITUTE

Recently, the molecular aspects of globin gene expression have met with even greater interest as researchers have attempted to use genetic engineering to produce a synthetic blood that would avoid the pitfalls of donor generated blood. In 1988, between 10 million and 14 million units of blood were used in the United States alone (Andrews, February 18, 1990, New York Times), an enormous volume precariously dependent on volunteer blood donations. About 5 percent of donated blood is infected by hepatitis virus (Id.) and, although screening procedures for HIV infection are generally effective, the prospect of contracting transfusion related A.I.D.S. remains a much feared possibility. Furthermore, transfused blood must be compatible with the blood type of the transfusion recipient; the donated blood supply may be unable to provide transfusions to individuals with rare blood types. In contrast, hemoglobin produced by genetic engineering would not require blood type matching, would be virus-free, and would be available in potentially unlimited amounts. Several research groups have explored the possibility of expressing hemoglobin in microorganisms. For example, see International Application No. PCT/US88/01534 by Hoffman and Nagai, which presents, in working

examples, production of human globin protein in E. coli.

2.4. TRANSGENIC ANIMALS

A transgenic animal is a non-human animal containing at least one foreign gene, called a transgene, in its genetic material. Preferably, the transgene is contained in the animal's germline such that it can be transmitted to the animal's offspring. A number of techniques may be used to introduce the transgene into an animal's genetic material, including, but not limited to, microinjection of the transgene into pronuclei of fertilized eggs and manipulation of embryonic stem cells (U.S. Patent No. 4,873,191 by Wagner and Hoppe; Palmiter and Brinster, 1986, Ann. Rev. Genet. 20:465-499; French Patent Application 2593827 published August 7, 1987). Transgenic animals may carry the transgene in all their cells or may be genetically mosaic.

Although the majority of studies have involved transgenic mice, other species of transgenic animal have also been produced, such as rabbits, sheep, pigs (Hammer et al., 1985, Nature 315:680-683) and chickens (Salter et al., 1987, Virology 157:236-240). Transgenic animals are currently being developed to serve as bioreactors for the production of useful pharmaceutical compounds (Van Brunt, 1988, Bio/Technology 6:1149-1154; Wilmut et al., 1988, New Scientist (July 7 issue) pp. 56-59).

Methods of expressing recombinant protein via transgenic livestock have an important theoretical advantage over protein production in recombinant bacteria and yeast; namely, the ability to produce large, complex proteins in which post-translational modifications, including glycosylation, phosphorylation, subunit assembly, etc. are critical for the activity of the molecule.

In practice, however, the creation of transgenic livestock has proved problematic. Not only is it technically difficult to produce transgenic embryos, but mature transgenic animals that produce significant quantities of recombinant protein may prove inviable. In pigs in particular, the experience has been that pigs carrying a growth hormone encoding transgene (the only transgene introduced into pigs prior to the present invention) suffered from a number of health problems, including severe arthritis, lack of coordination in their rear legs, susceptibility to stress, anoestrus in gilts and lack of libido in boars (Wilmut et al., supra). This is in contrast to transgenic mice carrying a growth hormone transgene, which appeared to be healthy (Palmiter et al., 1982, Nature 300:611-615). Thus, prior to the present invention, healthy transgenic pigs (which efficiently express their transgene(s)) had not been produced.

2.5. EXPRESSION OF GLOBIN GENES IN TRANSGENIC ANIMALS

Transgenic mice carrying human globin transgenes have been used in studying the molecular biology of globin gene expression. A hybrid mouse/human adult beta globin gene was described by Magram et al. in 1985 (Nature 315:338-340). Kollias et al. then reported regulated expression of human gamma-A, beta, and hybrid beta/gamma globin genes in transgenic mice (1986, Cell 46:89-94). Transgenic mice expressing human fetal gamma globin were studied by Enver et al. (1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037) and Constantoulakis et al. (1991, Blood 77:1326-1333). Autonomous developmental control of human embryonic globin gene switching in transgenic mice was observed by Raich et al. (1990, Science 250:1147-1149).

Transgenic mouse models for a variety of disorders of hemoglobin or hemoglobin expression have

been developed, including sickle cell disease (Rubin et al., 1988, Am. J. Human Genet. 42:585-591; Greaves et al., 1990, Nature 343:183-185; Ryan et al., 1990, Science 247:566-568; Rubin et al., 1991, J. Clin. Invest. 87:639-647); thalassemia (Anderson et al., 1985, Ann. New York Acad. Sci. (USA) 445:445-451; Sorenson et al., 1990, Blood 75:1333-1336); and hereditary persistence of fetal hemoglobin (Tanaka et al., 1990, Ann. New York Acad. Sci. (USA) 612:167-178).

Concurrent expression of human alpha and beta globin has led to the production of human hemoglobin in transgenic mice (Behringer et al., 1989, Science 245:971-973; Townes et al., 1989, Prog. Clin. Biol. Res. 316A:47-61; Hanscombe et al., 1989, Genes Dev. 3:1572-1581). It was observed by Hanscombe et al. (supra) that transgenic fetuses with high copy numbers of a transgene encoding alpha but not beta globin exhibited severe anemia and died prior to birth. Using a construct with both human alpha and beta globin genes under the control of the beta globin LCR, live mice with low copy numbers were obtained (Id.). Metabolic labeling experiments showed balanced mouse globin synthesis, but imbalanced human globin synthesis, with an alpha/beta biosynthetic ratio of about 0.6 (Id.).

3. SUMMARY OF THE INVENTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin and/or human globin. It is based, at least in part, on the discovery that transgenic pigs may be generated that express human hemoglobin in their erythrocytes and are healthy, suffering no deleterious effects as a result of heterologous hemoglobin production.

In particular embodiments, the present invention provides for transgenic pigs that express

human globin genes. Such animals may be used as a particularly efficient and economical source of human hemoglobin, in light of (i) the relatively short periods of gestation and sexual maturation in pigs; (ii) the size and frequency of litters, (iii) the relatively large size of the pig which provides proportionately large yields of hemoglobin; and (iv) functional similarities between pig and human hemoglobins in the regulation of oxygen binding affinity which enables the transgenic pigs to remain healthy in the presence of high levels of human hemoglobin.

The present invention also provides for recombinant nucleic acid constructs that may be used to generate transgenic pigs. In preferred embodiments, such constructs place the human alpha and beta globin genes under the same promoter so as to avoid deleterious effects of globin chain imbalance and/or titration of transcription factors due to constitutive β-globin promoter activity in an inappropriate cell type (e.g. a primitive erythrocyte). In other preferred embodiments of the invention, the constructs comprise the pig adult beta globin gene regulatory region, comprising the promoter or the 3' region of the pig beta globin gene.

In an additional embodiment, the present invention provides for a hybrid hemoglobin that comprises human α globin and pig β globin. The whole blood from transgenic pigs expressing this hybrid hemoglobin appears to exhibit a P_{50} that is advantageously higher than that of native human or pig blood.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as

to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells; and (iv) subjecting the released contents of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin. In a preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

4. DESCRIPTION OF THE FIGURES

Figure 1. Recombinant nucleic acid constructs. A. Construct $\alpha\alpha\beta$ (the "116 construct"); B. Construct $\alpha p\beta$ (the "185" construct); C. Construct $\beta p\alpha$ (the "290" construct); D. Construct $\epsilon p\zeta\beta\alpha$; E. Construct $\zeta p\epsilon\alpha p\beta$; F. Construct $\alpha p\beta$ carrying a $\beta 108$ Asn -> Asp mutation (the "hemoglobin Yoshizuka construct"); G. Construct $\alpha p\beta$ carrying a $\beta 108$ Asn -> Lys mutation (the "hemoglobin Presbyterian construct"); H. Construct $\alpha p\beta(\Delta\alpha)$ coinjected with LCR α (the "285" construct); I. Construct $\alpha p\beta$ carrying an $\alpha 134$ Thr -> Cys mutation (the "227" construct); J. Construct $\alpha p\beta$ carrying an $\alpha 104$ Cys-> Ser mutation (the "227" construct), a $\beta 93$ Cys -> Ala mutation, and a $\beta 112$ Cys -> Val mutation (the "228" construct); K. Construct $\alpha p\delta$ (the "263" construct); and L. Construct $\alpha p\delta(\Delta\alpha)$ coinjected with LCR α (the "274" construct); M. Construct LCR α coinjected with LCR $\epsilon\beta$ (the "240" construct); N. Construct $\alpha p\beta$ carrying a $\beta 61$ Lys -> Met mutation (the "Hemoglobin Bologna" construct); O. Construct LCR $\epsilon\alpha\beta$ (the "318" construct); P. Construct LCR $\alpha\epsilon\beta$ (the "319" construct); Q. Construct LCR $\alpha\alpha\epsilon\beta$ (the "329" construct); R. Construct LCR $\alpha\epsilon(^{Pig}\beta p)\beta$ (the

"339" construct); S. Construct αpβ carrying an α75 Asp -> Cys mutation (the "340" construct); T. Construct αpβ carrying an α42 Tyr -> Arg mutation (the "341" construct); U. Construct LCR ϵβαα (the "343" construct); V. Construct LCR ϵβα (the "347" construct); W. Construct αpβ carrying an α42 Tyr -> Lys mutation; X. Construct αpβ carrying an α42 Tyr -> Arg mutation; and a β99 Asp -> Glu mutation; Y. Construct αpβ carrying an α42 Tyr -> Lys mutation; and a β99 Asp -> Glu mutation.

Figure 2. Transgenic pig.

Figure 3. Demonstration of human hemoglobin expression in transgenic pigs. A. Isoelectric focusing gel analysis. B. Triton-acid urea gel of hemolysates of red blood cells representing human blood (lane 1); blood from transgenic pig 12-1 (lane 2), 9+3 (lane 3), and 6-3 (lane 4); and pig blood (lane 5) shows under-expression of human β globin relative to human α globin in the transgenic animals.

Figure 4. Separation of human hemoglobin and pig hemoglobin by DEAE chromatography. A. Hemolyzed mixture of human and pig red blood cells; B. Hemolysate of red blood cells collected from transgenic pig 6-3. C. Human and mouse hemoglobin do not separate by DEAE chromatography under these conditions. D. Isoelectric focusing of human hemoglobin purified from pig hemoglobin.

Figure 5. Isoelectric focussing gel of reassociated pig hemoglobin (lane 1); reassociated pig/human hemoglobin mixture (lanes 2 and 4); reassociated human hemoglobin (lane 3); and transgenic pig hemoglobin (lane 5).

Figure 6. Separation of human hemoglobin by QCPI chromatography.

Figure 7. Oxygen affinity of transgenic hemoglobin.

Figure 8. DNA sequence of the pig adult beta globin gene regulatory region, including the promoter region. Sequence extending to 869 base pairs upstream of the ATG initiator codon (boxed) of the pig beta globin gene is shown. The position of the initiation of mRNA, the cap site, is indicated by an arrow. The sequences corresponding to GATA transcription factor binding sites are underlined.

Figure 9. Comparison of pig (top) and human (bottom) beta globin regulatory sequences. Differences in the two sequences are marked by asterisks.

Figure 10. Graph depicting the percent homology between pig and human adult beta globin gene regulatory sequences, with base pair distance from the initiator codon mapped on the abscissa. A comparison of mouse and human sequences is also shown (dotted line with error bar).

Figure 11. Map of plasmid pgem5/PigβPr(k) which contains the DNA sequence depicted in Figure 8.

Figure 12. Representation of the 339 and 354 cassettes for the production of human hemoglobin in transgenic pigs.

Figure 13. Map of plasmid pSaf/Pigϵ(k), containing the pig ϵ gene.

Figure 14. Representation of the 426 and 427 expression cassettes for the production of ϵ^{Pig}, β^{human} and α^{human} hemoglobins in transgenic pigs.

Figure 15. Iso-electric focussing gel of hemoglobin produced by transgenic pig 70-3, which carries the 339 construct, and by transgenic pig 6-3, which carries the 116 construct. Human hemoglobin is run as a standard.

Figure 16. Map of plasmid pig3'β containing the 3' end of the pig beta globin gene.

Figure 17. Transgenic pigs obtained from construct "339" (See Figure 1R). Levels of human hemoglobin expression and copy number are shown.

Figure 18. Isolelectric focussing gel of hemoglobin levels in transgenic pigs obtained using construct "339".

Figure 19. Isoelectric focussing gel demonstrating levels of hemoglobin expression in representative transgene positive 38-4 offspring carrying the "185" construct (or αpβ construct; see Figure 1B).

Figure 20. Molecular modeling of hybrid human α/pig β and human α/human β hemoglobin molecules. β subunits are in blue, α subunits in red. Above the middle helix of the β human (blue) one can see a gap in the green contour (see arrow). In the hybrid this gap is filed in. This difference is due to a change at β112 Cys-->Val, where Valine contributes to greater hydrophobic interactions.

Figure 21. Molecular modeling demonstrating the differences at the $\alpha_1\beta_1$ interface between a β globin containing Cys at position 112 (the yellow molecule) and a β globin with Val at position 112 (the white molecule). Cys is yellow, Val is white and the opposing α interface is red. Val is flexible. One arm of its branch can easily move for a nearly perfect fit against the α subunit residues. The yellow Cys is slightly further allowing for a small gap (see arrow). Biosyn's standard default Van der Waal's distance was used.

Figure 22. Purification of Hb Presbyterian from transgenic pig hemosylate.

Figure 23. Characterization of purified Hb Presbyterian by HPLC showing separation of the heme moiety, pig α globin ("p alpha"), human beta globin ("h beta"), human alpha globin ("h alpha") and pig beta globin ("p beta").

Figure 24. Oxygen binding curve for Hb Presbyterian.

Figure 25. Purification of Hb Yoshizuka from transgenic pig hemolysate.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a method of producing human hemoglobin that utilizes transgenic pigs, novel globin-encoding nucleic acid constructs, and transgenic pigs that express human hemoglobin. For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) preparation of globin gene constructs;

(ii) preparation of transgenic pigs;

(iii) preparation of human hemoglobin and its separation from pig hemoglobin; and

(iv) preparation of human/pig hybrid hemoglobin.

5.1. PREPARATION OF GLOBIN GENE CONSTRUCTS

The present invention provides for a method of producing human globin and/or hemoglobin in transgenic pigs. Human hemoglobin is defined herein to refer to hemoglobin formed by globin chains encoded by human globin genes (including alpha, beta, delta, gamma, epsilon and zeta genes) or variants thereof which are naturally occurring or the products of genetic engineering. Such variants are at least about ninety percent homologous in amino acid sequence to a naturally occurring human hemoglobin. In preferred embodiments, the human hemoglobin of the invention comprises a human alpha globin and a human beta globin chain. The human hemoglobin of the invention comprises at least two different globin chains, but may comprise more than two chains, to form, for example, a tetrameric molecule, octameric molecule, etc. In preferred embodiments of the invention, human

hemoglobin consists of two human alpha globin chains and two human beta globin chains. As discussed infra, the present invention also provides for hybrid hemoglobins comprising human α globin and pig β globin.

According to particular embodiments of the present invention, at least one human globin gene, such as a human alpha and/or a human beta globin gene, under the control of a suitable promoter or promoters, is inserted into the genetic material of a pig so as to create a transgenic pig that carries human globin in at least some of its red blood cells. This requires the preparation of appropriate recombinant nucleic acid sequences. In preferred embodiments of the invention, both human α and human β genes are expressed. In an alternative embodiment, only human α globin or human β globin is expressed. In further embodiments, human embryonic or fetal globin genes are expressed or are used as developmental expression regulators of adult genes.

Human alpha and beta globin genes may be obtained from publicly available clones, e.g. as described in Swanson et al., 1992, Bio/Technol. 10:557-559. Nucleic acid sequences encoding human alpha and beta globin proteins may be introduced into an animal via two different species of recombinant constructs, one which encodes human alpha globin, the other encoding human beta globin; alternatively, and preferably, both alpha and beta-encoding sequences may be comprised in the same recombinant construct. The pig epsilon globin gene is contained in plasmid psaf/pig ε (k) (Figure 13), deposited with the ATCC and assigned accession number 75373.

A suitable promoter, according to the invention, is a promoter which can direct transcription of human alpha and/or beta globin genes in red blood cells. Such a promoter is preferably

selectively active in erythroid cells. This would include, but is not limited to, a globin gene promoter, such as the human alpha, beta, delta, epsilon or zeta promoters, or a globin promoter from another species. It may, for example, be useful to utilize pig globin promoter sequences. For example, as discussed in Section 10, infra, the use of the endogenous pig β globin gene control region, as contained in plasmid Pgem5/Pigβpr(K), deposited with the ATCC and assigned accession number 75371 and having the sequence set forth in Figure 8, has been shown to operate particularly efficiently. The human alpha and beta globin genes may be placed under the control of different promoters, but, since it has been inferred that vastly different levels of globin chain production may result in lethality, it may be preferable to place the human alpha and beta globin genes under the control of the same promoter sequence. In order to avoid chain imbalance and/or titration of transcription factors due to constitutive β-globin promoter activity in an inappropriate cell type, it is desirable to design a construct which leads to coordinate expression of human alpha and beta globin genes at the same time in development and at quantitatively similar levels.

In one particular, non-limiting embodiment of the invention, a construct comprising the $\alpha\alpha\beta$ construct (also termed the "116" construct; Swanson et al., 1992, Bio/Technol. 10:557-559; see Figure 1A) may be utilized. Although this construct, when present as a transgene at high copy number, has resulted in deleterious effects in mice, it has been used to produce healthy transgenic pigs (see Example Section 6, infra).

In another particular, non-limiting embodiment of the invention, a construct comprising the $\alpha p\beta$ sequence (also termed the "185" construct; see

Figure 1B) may be used. Such a construct has the advantage of placing both alpha and beta globin-encoding sequences under the control of the same promoter (the alpha globin promoter).

In another particular, non-limiting embodiment of the invention, a construct coding for di-alpha globin like polypeptides may be introduced to form transgenic pigs that produce human hemoglobins with decreased dimerization and an increased half-life (WO Patent 9013645).

In yet another particular, non-limiting embodiment of the invention, a construct comprising the human adult alpha globin and epsilon globin gene, the pig beta globin gene control region and the human beta globin gene (the "339 construct, see Figure 1R) may be used.

Furthermore, the incorporation of a human or pig epsilon globin gene into the construct may facilitate the production of high hemoglobin levels. The pig epsilon globin gene may permit correct developmental regulation of the adult β globin gene. High levels of expression of introduced adult alpha globin gene(s) may result in a chain imbalance problem during intrauterine development of a transgenic pig embryo (because an adult beta globin gene in the construct would not yet be expressed) thereby compromising the viability of the embryo. By providing high levels of embryonic globins during development, the viability of such embryos may be improved. The pig epsilon globin gene, as contained in plasmid pSaf/Pige, deposited with the ATCC and assigned accession number 75373, is shown in Figure 13.

The present invention, in further specific embodiments, provides for (i) the construct $\beta p\alpha$, in which the human alpha and beta globin genes are driven by separate copies of the human beta globin promoter

(Figure 1C); (ii) the $\epsilon p\zeta\beta p\alpha$ construct, which comprises human embryonic genes zeta and epsilon under the control of the epsilon promoter and both alpha and beta genes under the control of the beta promoter (Figure 1D); (iii) the $\zeta p\epsilon\alpha p\beta$ construct, which comprises human embryonic genes zeta and epsilon under the control of the zeta promoter and both alpha and beta genes under the control of the alpha promoter (Figure 1E); (iv) the $\alpha p\beta$ construct carrying a mutation that results in an aspartic acid residue (rather than an asparagine residue) at amino acid number 108 of β globin protein, to produce hemoglobin Yoshizuka (Figure 1F, construct "294"); (v) the $\alpha p\beta$ construct carrying a mutation that results in a lysine residue (rather than an asparagine residue) at amino acid number 108 of β-globin protein, to produce hemoglobin Presbyterian (Figure 1G, construct "293"); (vi) the $\alpha p\beta(\Delta\alpha)$ construct, coinjected with LCR α, which comprises the human β-globin gene under the control of the human α-globin promoter and a separate nucleic acid fragment comprising the human α-globin gene under its own promoter (Figure 1H); (vii) the $\alpha p\beta$ construct carrying a mutation that results in a cysteine residue (rather than a threonine residue) at amino acid number 134 of α-globin protein (Figure 1I); (viii) the $\alpha p\beta$ construct carrying a mutation that results in a serine residue (rather than a cysteine residue) at amino acid number 104 of the α-globin protein, an alanine residue (rather than a cysteine residue) at amino acid number 93 of the β-globin protein and a valine residue (rather than a cysteine residue) at amino acid number 112 of the β-globin protein (Figure 1J); (ix) the $\alpha p\delta$ construct, which comprises the human adult α-globin promoter under its own promoter and the human δ-globin gene under the control of the human adult α-globin promoter (Fig. 1K); (x) Construct $\alpha p\delta(\Delta\alpha)$ coinjected with LCR α,

which comprises the human δ-globin gene under the control of the human α-globin promoter and a separate nucleic acid fragment comprising the human α-globin gene under its own promoter (Fig. 1L); (xi) construct LCR α coinjected with LCR εβ, which comprises the human α-globin gene under the control of its own promoter and a separate nucleic acid fragment comprising the human embryonic ε-globin gene and the adult β-globin gene under the control of their own promoters (Fig. 1M); (xii) the αpβ construct carrying a mutation that results in a methionine residue (rather than a lysine residue) at amino acid number 61 of the α-globin protein (Fig. 1N); (xiii) the εαβ construct, which comprises the human embryonic epsilon gene, the human adult alpha globin gene and the human adult beta globin gene linked in tandem from 5' to 3' (Fig. 1O); (xiv) the αεβ construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1P); (xv) the ααεβ construct, which comprises two copies of the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1Q); (xvi) the αε(Pβp)β construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene under the control of the endogenous porcine adult beta globin promoter all linked in tandem from 5'- to 3' (Fig. 1R); (xvii) the αpβ construct carrying a mutation that results in a cysteine residue (rather than an aspartic acid residue) at amino acid number 75 of the α-globin protein (Fig. 1S); (xviii) the αpβ construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the α-globin protein (Fig. 1T); (xvix) the LCR εβαα construct, which comprises

the human embryonic epsilon globin gene, the human adult beta globin gene and two copies of the human adult alpha-globin gene linked in tandem from 5'- to 3' (Fig. 1U); (xx) the LCR $\epsilon\beta\alpha$ construct, which comprises the human embryonic epsilon globin gene, the human adult beta globin gene and the human adult alpha-globin gene linked in tandem from 5'- to 3' (Fig. 1V); (xxi) the $\alpha p\beta$ construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the α-globin protein (Fig. 1W); (xxii) the $\alpha p\beta$ construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the α-globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the β-globin protein (Fig. 1X); (xxiii) the $\alpha p\beta$ construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the α-globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the β-globin protein (Fig. 1Y); and (xxiv) the $\alpha^{pig}\epsilon(^{pig}\beta p)\beta$ construct comprising the pig epsilon globin gene and beta globin control region (constructs 426 and 427, Figure 14).

In transgenic pigs expressing human hemoglobin three types of hemoglobin dimers are detectable: pig α/pig β, human α/human β, and hybrid human α/pig β. In certain embodiments of the invention, it may be desirable to decrease the amount of hybrid hemoglobin. Accordingly, the molecular basis for the formation of hybrid hemoglobin has been investigated using molecular modeling studies. Based on the information derived from these studies, the human alpha and beta globin structures can be modified to increase the level of human α/human β dimers (See Section 11.), so that in further embodiments of the

invention, constructs comprising the αpβ sequence may be modified to code for α or β globin proteins carrying amino acid changes that will lead to increases in the level of human α/human β hemoglobin dimers in transgenic pigs. The present invention, provides for constructs which encode human α globin and human β globin carrying one or more of the following mutations in the α globin molecule: (1) a Thr at position 30 instead of Glu; (ii) a Tyr at position 36 instead of Phe; (iii) a Phe instead of Leu at position 106; (iv) a Ser or Cys instead of Val at position 107; and/or (v) a Cys instead of Ala at position 111. In specific embodiments, the construct carrying such mutation(s) is the αpβ construct. The present invention, in further embodiments, provides for constructs which encode human α globin and human β globin carrying one or more of the following mutations in the β globin molecule: (1) a Leu instead of Val at position 33; (ii) a Val or Ile instead of Cys at position 112; (iii) a Val or Leu instead of Ala at position at position 115; (iv) a His instead of Gly at position 119; (v) a Met instead of Pro at position 125; (vi) an Ile instead of Ala at position 128; and/or (vii) a Glu instead of Gln at position 131; and/or (viii) a Glu instead of Gln at position 131. In specific embodiments, the construct carrying the mutation(s) is the αpβ construct.

In further embodiments it may be desirable to include, in constructs, the untranslated 3' end of the pig beta globin gene as contained in plasmid pPig3'β (Figure 16) as deposited with the ATCC and assigned accession number 75372. (see, for example, construct 354 in Figure 12 and Figures 426 and 427 in Figure 14). Such constructs may also be useful in the expression of non-globin protein in pig erythrocytes.

In further embodiments, the pig beta globin control region depicted in Figures 8 and 9 may be used

in constructs that encode non-globin proteins for the expression of said proteins in transgenic pig or other non-human erythrocytes.

The recombinant nucleic acid constructs described above may be inserted into any suitable plasmid, bacteriophage, or viral vector for amplification, and may thereby be propagated using methods known in the art, such as those described in Maniatis et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. In the working examples presented below, the pUC vector (Vanish-Perron et al., 1985, Gene 103-119) was utilized.

The present invention further provides for isolated and purified nucleic acids comprising the pig adult beta globin promoter regulatory region, the pig 3' beta globin region, and the pig epsilon globin gene as comprised, respectively, in plasmids pgem5/Pigβpr(K) (ATCC accession no. 75371), ppig3'β (ATCC accession no. 75372), and pSaf/pigϵ(k) (ATCC accession no. 75373), respectively.

Constructs may desirably be linearized for preparation of transgenic pigs. Vector sequence may desirably be removed.

5.2. PREPARATION OF TRANSGENIC PIGS

The recombinant constructs described above may be used to produce a transgenic pig by any method known in the art, including but not limited to, microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection, transduction, retroviral infection, etc. Species of constructs may be introduced individually or in groups of two or more types of construct.

According to a preferred specific embodiment of the invention, a transgenic pig may be produced by the methods as set forth in Example Section 6, infra. Briefly, estrus may be synchronized in sexually mature

gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts may be given an intramuscular injection (IM) of prostaglandin $F_{2\alpha}$ (Lutalyse: 10 mg/injection) at 0800 and 1600 hours. Twenty-four hours after the last day of AT consumption all donor gilts may be administered a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) may be administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts may be checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration may be bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG one- and two-cell ova may be surgically recovered from bred donors using the following procedure. General anesthesia may be induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract may be exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) may be inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova may be flushed in retrograde fashion by inserting a 20g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) may be infused into the oviduct and flushed toward the glass cannula. The medium may be collected into sterile 17 x 100 mm polystyrene tubes.

Flushings may be transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova may be washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 µl drops of BMOC-3 medium under oil. Ova may be stored at 38.5°C under a 90% N_2, 5% O_2, 5% CO_2 atmosphere until microinjection is performed.

One- and two-cell ova may be placed in a Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova may then be transferred to a 5 - 10 µl drop of HEPES medium under oil on a depression slide. Microinjection may be performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (linearized at a concentration of about 1ng/µl of Tris-EDTA buffer) may be injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova may be returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N_2, 5% CO_2, 5% O_2 atmosphere prior to their transfer to suitable recipients. Ova may preferably be transferred within 10 hours of recovery.

Only recipients which exhibit estrus on the same day or 24 hours later than the donors may preferably be utilized for embryo transfer. Recipients may be anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one-and/or two-cell ova and 4-6 control ova may be transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set may be connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium may be aspirated into the tubing. The tubing may then be fed through the ostium of the

oviduct until the tip reaches the lower third or isthmus of the oviduct. The ova may be subsequently expelled as the tubing is slowly withdrawn.

The exposed portion of the reproductive tract may be bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin may be sutured as three separate layers. An uninterrupted Halstead stitch may be used to close the lina alba. The fat and skin may be closed using a simple continuous and mattress stitch, respectively. A topical antibacterial agent (e.g. Furazolidone) may then be administered to the incision area.

Recipients may be penned in groups of about four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients may be checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection may be performed using ultrasound. On day 107 of gestation recipients may be transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing may be induced by the administration of prostaglandin $F_{2\alpha}$ (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients may be expected to farrow within 34 hours following PGF2a administration.

Twenty-four hours after birth, all piglets may be processed, i.e. ears notched, needle teeth clipped, 1 cc of iron dextran administered, etc. A tail biopsy and blood may also be obtained from each pig.

Pigs produced according to this method are described in Example Section 6, infra, and are depicted in Figure 2. Such pigs are healthy, do not appear to be anemic, and appear to grow at a rate comparable to that of their non-transgenic

littermates. Such pigs may transmit the transgene to their offspring.

Pigs having certain characteristics may be especially useful for the production of human hemoglobin; such pigs, examples of which follow, represent preferred, non-limiting, specific embodiments of the invention.

According to one preferred specific embodiment of the invention, a transgenic pig contains at least twenty copies of a globin transgene.

According to a second preferred specific embodiment, the P_{50} of whole blood of a transgenic pig according to the invention is increased by at least ten percent over the P_{50} of the whole blood of a comparable non-transgenic pig, taking into consideration factors such as altitude, oxygen concentrations, pregnancy, the presence of mutant hemoglobin, etc. Thus, the present invention provides for a non-pregnant transgenic pig that carries and expresses a human globin transgene in which the P_{50} of whole blood of the transgenic pig is at least ten percent greater than the P_{50} of whole blood of a comparable non-pregnant non-transgenic pig at the same altitude.

In other preferred specific embodiments, the present invention provides for a transgenic pig in which the amount of human globin produced relative to total hemoglobin is at least two percent, more preferably at least five percent, and most preferably at least ten percent.

Section 6, infra, describes transgenic pigs which serve as working examples of preferred, non-limiting, specific examples of the invention.

5.3. PREPARATION OF HUMAN HEMOGLOBIN AND ITS SEPARATION FROM PIG HEMOGLOBIN

The present invention provides for a method for producing human hemoglobin comprising introducing a transgene or transgenes encoding human hemoglobin, such as a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its blood cells.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells to form a lysate; (iv) subjecting the lysate of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin; and (v) collecting the fractions that contain purified human hemoglobin. Such fractions may be identified by isoelectric focusing in parallel with appropriate standards. In a preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

In order to prepare human hemoglobin from the transgenic pigs described above, red blood cells are obtained from the pig using any method known in the art. The red blood cells are then lysed using any method, including hemolysis in a hypotonic solution such as distilled water, or using techniques as described in 1981, Methods in Enzymology Vol. 76, and/or tangential flow filtration.

For purposes of ascertaining whether human hemoglobin is being produced by a particular

transgenic pig, it may be useful to perform a small-scale electrophoretic analysis of the hemolysate, such as, for example, isoelectric focusing using standard techniques.

Alternatively, or for larger scale purification, human hemoglobin may be separated from pig hemoglobin using ion exchange chromatography. Surprisingly, as discussed in Section 7, supra, human hemoglobin was observed to readily separate from pig hemoglobin using ion exchange chromatography whereas mouse hemoglobin and human hemoglobin were not separable by such methods. Any ion exchange resin known in the art or to be developed may be utilized, including, but not limited to, resins comprising diethylaminoethyl, Q-Sepharose, QCPI (I.B.F.) Zephyr, Spherodex, ectiola, carboxymethylcellulose, etc., provided that the resin results in a separation of human and pig hemoglobin comparable to that achieved using DEAE resin.

According to a specific, nonlimiting embodiment of the invention, in order to separate human from pig hemoglobin (including human/pig hemoglobin hybrids) to produce substantially pure human hemoglobin, a hemolysate of transgenic pig red blood cells, prepared as above may be applied to a DEAE-anion exchange column equilibrated with 0.2 M glycine buffer at Ph 7.8 and washed with 0.2 M glycine Ph 7.8/5 Mm NaCl, and may then be eluted with a 5-30 Mm NaCl gradient, or its equivalent (see, for example, Section 9 infra). Surprisingly, despite about 85 percent homology between human and pig globin chains, human and pig hemoglobin separates readily upon such treatment, with human hemoglobin eluting earlier than pig hemoglobin. Elution may be monitored by optical density at 405 nm and/or electrophoresis of aliquots taken from serial fractions. Pig hemoglobin, as well as tetrameric hemoglobin composed of heterodimers

formed between pig and human globin chains, may be separated from human hemoglobin by this method. Human hemoglobin produced in a transgenic pig and separated from pig hemoglobin by this method has an oxygen binding capability similar to that of native human hemoglobin.

According to another specific, non-limiting embodiment of the invention, human hemoglobin may be separated from pig hemoglobin (including human/pig hemoglobin hybrids) using QCPI ion exchange resin as follows:

About 10 mg of hemoglobin prepared from transgenic pig erythrocytes may be diluted in 20ml of Buffer A (Buffer A = 10mM Tris, 20mM Glycine Ph 7.5). This 20ml sample may then be loaded at a flow rate of about 5ml/min onto a QCPI column (10 ml) which has been equilibrated with Buffer A. The column may then be washed with 2 volumes of Buffer A, and then with 20 column volumes of a 0-50mM NaCl gradient (10 column volumes of Buffer A + 10 column volumes of 10mM Tris, 20mM Glycine, 50mM NaCl, Ph 7.5) or, alteratively, 6 column volumes of 10mM Tris, 20mM Glycine, 15mM NaCl, pH 7.5, and the $O.D._{280}$ absorbing material may be collected in fractions to yield the separated hemoglobin; human hemoglobin being identified, for example, by isoelectric focusing using appropriate standards. The QCPI column may be cleaned by elution with 2 column volumes of 10mM Tris, 20mM Glycine, 1M NaCl, pH 7.5.

For certain mutant hemoglobins, it may be desirable to utilize a modified purification procedure. Accordingly, for the separation of Hb Presbyterian from pig Hb, a procedure as described in Example Section 12.1, infra, may be used, and for separation of Hb Yoshizuka, a procedure as described in Example Section 12.2, infra, may be used.

5.4. PREPARATION OF HUMAN/PIG HYBRID HEMOGLOBIN

The present invention also provides for essentially purified and isolated human/pig hybrid hemoglobin, in particular human α/pig β hybrid hemoglobin. Pig α/human β hybrid has not been observed to form either in vitro in reassociation experiments or in vivo in transgenic pigs.

The present invention provides for hybrid hemoglobin and its use as a blood substitute, and for a pharmaceutical composition comprising the essentially purified and isolated human/pig hemoglobin hybrid in a suitable pharmacological carrier.

Hybrid hemoglobin may be prepared from transgenic pigs, as described herein, and then purified by chromatography, immunoprecipitation, or any other method known to the skilled artisan. The use of isoelectric focusing to separate out hemoglobin hybrid is shown in Figures 3 and 5.

Alternatively, hybrid hemoglobin may be prepared using nucleic acid constructs that comprise both human and pig globin sequences which may then be expressed in any suitable microorganism, cell, or transgenic animal. For example, a nucleic acid construct that comprises the human α and pig β globin genes under the control of a suitable promoter may be expressed to result in hybrid hemoglobin. As a specific example, human α globin and pig β globin genes, under the control of cytomegalovirus promoter, may be transfected into a mammalian cell such as a COS cell, and hybrid hemoglobin may be harvested from such cells. Alternatively, such constructs may be expressed in yeast or bacteria.

It may be desirable to modify the hemoglobin hybrid so as to render it non-immunogenic, for example, by linkage with polyethylene glycol or by encapsulating the hemoglobin in a membrane, e.g. in a liposome.

6. EXAMPLE: GENERATION OF TRANSGENIC PIGS THAT PRODUCE HUMAN HEMOGLOBIN

6.1. MATERIALS AND METHODS

6.1.1. NUCLEIC ACID CONSTRUCTS

Constructs 116 (the $\alpha\alpha\beta$ construct), 185 (the $\alpha p\beta$ construct), 263 (the $\alpha p\delta$ construct) 339, 293 and 294 were microinjected into pig ova as set forth below in order to produce transgenic pigs.

6.1.2. PRODUCTION OF TRANSGENIC PIGS

Estrus was synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts received an intramuscular injection (IM) of prostaglandin $F_{2\alpha}$ (Lutalyse: 10 mg/injection) at 0800 and 1600. Twenty-four hours after the last day of AT consumption all donor gilts received a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) was administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts were checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration were bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG, one- and two-cell ova were surgically recovered from bred donors using the following procedure. General anesthesia was induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract was exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) was inserted into the ostium of the

oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova were flushed in retrograde fashion by inserting a 20 g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) was infused into the oviduct and flushed toward the glass cannula. The medium was collected into sterile 17 x 100 mm polystyrene tubes. Flushings were transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova were washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 µl drops of BMOC-3 medium under oil. Ova were stored at 38°C under a 90% N_2, 5% O_2, 5% CO_2, atmosphere until microinjection was performed.

One- and two-cell ova were placed in an Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova were then transferred to a 5 -10 µl drop of HEPES medium under oil on a depression slide. Microinjection was performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (1ng/µl of Tris-EDTA buffer) were injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova were returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N_2, 5% CO_2, 5% O_2 atmosphere prior to their transfer to suitable recipients. Ova were transferred within 10 hours of recovery.

Only recipients which exhibited estrus on the same day or 24 hours later than the donors were utilized for embryo transfer. Recipients were

anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one- and/or two-cell ova and 4-6 control ova were transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set was connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium were aspirated into the tubing. The tubing was then fed through the ostium of the oviduct until the tip reached the lower third or isthmus of the oviduct. The ova were subsequently expelled as the tubing was slowly withdrawn.

The exposed portion of the reproductive tract was bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin were sutured as three separate layers. An uninterrupted Halstead stitch was used to close the lina alba. The fat and skin were closed using a simple continuous and mattress stitch, respectively. A topical antibacterial agent (Furazolidone) was then administered to the incision area.

Recipients were penned in groups of four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients were checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection was performed using ultrasound. On day 107 of gestation recipients were transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing was induced by the administration of prostaglandin $F_{2\alpha}$ (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients farrowed within 34 hours following PGF2a administration.

Twenty-four hours after birth, all piglets were processed, i.e. ears were notched, needle teeth clipped, 1 cc of iron dextran was administered, etc.

A tail biopsy and blood were also obtained from each pig.

6.2. RESULTS AND DISCUSSION

Of 3566 injected ova, thirteen transgenic pigs that expressed human hemoglobin were born, two of which died shortly after birth due to normal breeding-related incidents completely unrelated to the fact that they were transgenic pigs (Table I). The remaining 11 appeared to be healthy. A photograph of one transgenic pig is presented in Figure 2. Profiles of the pigs and of the percent "authentic" and "hybrid" human hemoglobin ("HB") produced are set forth in Table II, infra. Total hemoglobin was calculated as the sum of human $\alpha\beta$ plus one-half of the human α pig β hybrid. Figure 3 presents the results of isoelectric focussing and triton acid urea gels of hemoglobin produced by three of these pigs (numbers 12-1, 9-3, and 6-3) which demonstrate the expression of human alpha and beta globin in these animals.

TABLE I

Efficiency of Transgenic Pig Production
Human Hemoglobin Gene Construct(s)

Parameter	Total After 22 Trials
Total Ova Collected	8276
Total # Fertilized	7156
Total # Injected	3566
# Injected Ova Transferred	3566
# Control Ova Transferred	279
# Recipients Used	104
# Pigs Born (Male, Female)	208,332
# Transgenic (Male, Female)	8,5 (0.36)[a]
# Expressing	13

[a] Proportion of injected ova which developed into transgenic pigs (13 transgenics/3566 injected ova).

TABLE II

FOUNDERS

PIG	GENDER	TRANSGENE CONSTRUCT	AUTHENTIC HUMAN HB	HYBRID HB	TOTAL HUMAN HB	COPY #
6-3	F	116	6.2%	8.1%	10.3%	57
9-3	F	116	1.0%	33.1%	16.6%	1
22-2	M	185	<1%	5.0%	5.0%	55
33-7	F	185	*died shortly after birth			0.5
38-1	F	185	1.0%	8.3%	5.2%	17
38-3	M	185	4.7%	17.2%	13.2%	22
38-4	M	185	3.2%	7.0%	6.7%	5
47-3	M	263	<1%	2.9%	2.0%	4-6
47-4	F	263	<1%	18.5%	10.0%	1-2
52-3	M	263	<1%	7.6%	4.0%	
52-7	M	263	<1%	26.4%	13.0%	
53-11	M	263	<1%	15.5%	8.0%	
70-3	F	339	23	31	38	3

Table III presents the profiles of offspring of pig number 9-3, which shows that the F1 generation of transgenic pigs are capable of expressing hemoglobin. Of note, none of the offspring of pig number 6-3 were found to be transgenic, possibly due to the absence of transgene in the animal's reproductive tissue.

Table IV presents hemoglobin expression data of offspring of pig 38-4 carrying the "185" construct (the "αpβ" construct; see Figure 1B). Table V presents a summary of the profiles of offspring of pig number 38-4 in which a large percentage (37.1%) of offspring were positive for expression of human hemoglobin indicating germ line transmission of the transgene. Figure 19 presents the results of isoelectric focussing which demonstrates the levels of hemoglobin expression in representative transgene positive 38-4 offspring.

TABLE III

F1 (OFFSPRING) OF PIG 9-3

PIG	GENDER	CONST.	AUTHENTIC HUMAN HB	HYBRID HUMAN HB	TOTAL HUM.	COPY #
9-3-1	F	116	1.0%	31.5%	16.0%	1
9-3-2*	F	116	1.0%	32.9%	17.0%	1
9-3-3	M	116	1.0%	29.7%	15.0%	1
9-3-4	M	116	1.0%	32.8%	17.0%	1
9-3-6	F	116	1.0%	29.1%	15.0%	1
9-3-8	M	116	1.0%	31.6%	16.0%	1
9-3-9	M	116	1.0%	30.2%	16.0%	1

*9-3-2 died the day after birth.

EXPRESSION LEVEL FEMALES EXPRESSION LEVEL

59 59

TABLE IV

EXPRESSION DATA PER LITTER FOR TRANSGENIC PIGS CARRYING THE "185" CONSTRUCT						
Founder	Litter No.	Gilt	Pigs	% Positive	#Tg	Avg. Authentic HbA
38-4	1	544	10	20.0%	2	8.8%
	2	213	11	45.4%	5	4.9%
	3	882	5	20.0%	1	10.9%
	4	4923	6	83.3%	5	9.4%
	5	710	6	75.0%	4	4.5%
	6	978	11	36.4%	4	7.1%
	7	466	4	25.0%	1	3.6%
	8	464	15	33.3%	5	5.1%
	9	461	8	62.5%	5	6.6%
	10	1657	10	30.0%	3	9.0%
	11	892	3	33.3%	1	5.7%
	12	995	11	27.3%	3	4.4%
	13	209	11	36.4%	4	5.4%
	14	424	10	30.0%	3	5.9%
	15	1659	14	35.7%	5	4.4%
	16	420	12	8.3%	1	2.0%
	17	373	7	28.6%	2	11.8%
	18	497	8	62.5%	5	6.0%

TABLE IV (CONT'D)

EXPRESSION DATA PER LITTER FOR TRANSGENIC PIGS CARRYING THE "185" CONSTRUCT						
Founder	Litter No.	Gilt	Pigs	% Positive	#Tg	Avg. Authentic HbA
	19	742	8	25.0%	2	1.0%
	20	1420	14	42.9%	6	8.1%
	21	41	5	40.0%	2	1.0%
	22	540	11	36.4%	4	5.3%
	23	7114	11	54.5%	6	3.4%
	24	744	11	27.3%	3	4.9%
	25	600	14	42.9%	6	5.5%
	26	1180	9	44.4%	4	2.0%
	27	1137	12	25.0%	3	6.1%
	28	970	8	37.5%	3	10.8%
	29	78	6	0	0	
	30	214	14	50.0%	7	5.5%
	31	279	6	50.0%	3	10.3%
	32	281	11	45.5%	5	5.1%
	33	21-474	6	33.3%	2	12.3%
	34	1151	10	30.0%	3	5.3%
			318		118	

TABLE V

38-4 BREEDING SUMMARY

FOUNDER	LITTERS	PIGLETS	PIGS/LITTER	TRANSGENIC	FREQUENCY	AVG. AUTHENTIC HbA
38-4(M)	34	318	9.4	118	37.1%	6.2%

MALES	AUTHENTIC HUMAN HB EXPRESSION LEVEL	FEMALES	AUTHENTIC HUMAN HB EXPRESSION LEVEL
59	5.7%	59	6.8%

The birth weights of the transgenic pigs have been approximately equivalent to the birth weights of their non-transgenic littermates. As the transgenic pigs matured, their weights remained comparable to the weights of control animals.

7. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY DEAE CHROMATOGRAPHY

7.1. MATERIALS AND METHODS

7.1.1. PURIFICATION BY DEAE CHROMATOGRAPHY

For purification, red blood cells were collected by centrifugation of 5000 rpm for 3 minutes in an eppendorf microcentrifuge and washed three times with an equal volume (original blood) of 0.9% NaCl. Red cells were lysed with 1.5 volumes deionized H_2O, centrifuged at 15,000 rpm, and the supernatant was fractionated by anion exchange chromatography. DEAE cellulose chromatography (DE-SE manufactured by Whatman, Ltd.) was performed according to W. A. Schroeder and T. H. J. Huisman "The Chromatography of Hemoglobin", Dekker, New York, pp. 74-77. The 0.25 ml red cell hemolysate described above was applied to 1 cm x 7 cm DE-52 column pre-equilibrated in 0.2 M glycine Ph 7.8 and was washed with 5 column volumes of 0.2 M glycine Ph 7.8/5 Mm NaCl. Hemoglobins were eluted with a 200 ml 5-30 mM NaCl/0.2 M glycine pH 7.8 gradient. To complete elution of pig hemoglobin, an additional 50 to 100 ml of 30 mM CaCl/glycine pH 7.8 was added to the column. Elution of hemoglobin was monitored by absorbance of 415 mM and by IEF analysis of column fractions.

7.1.2. REASSOCIATION OF GLOBIN CHAINS

Reassociation of globin chains was performed essentially as described in Methods in Enzymol. 76:126-133. 25 lambda of pig blood, 25 lambda of

human blood, or a 25 lambda mixture of 12.5 lambda human blood and 12.5 lambda pig blood were treated as follows. The blood was pelleted at a setting of 5 on microfuge for 2 minutes, then washed three times with 100 lambda 0.9 percent NaCl. The cells were lysed with 50 lambda H_2O, then spun at high speed to confirm lysis. 50 lambda of the lysed cells was then combined with 50 lambda 0.2 M Na Acetate, pH 4.5, put on ice and then incubated in a cold room overnight. After adding 1.9 ml 0.1 M NaH_2PO_4, pH 7.4 each sample was spun in centricon tubes at 4°C and 5K until about 0.5 ml remained. Then 1 ml of 0.1 M NaH_2PO_4 pH 7.4 was added and spun through at about 5K until about 0.2 ml volume was left. The hemoglobin was then washed from the walls of the centricon tube, an eppendorf adaptor was attached, and a table top microfuge was used to remove each sample from its centricon tube. The samples were then analyzed by isoelectric focusing.

7.2. RESULTS AND DISCUSSION

7.2.1. HUMAN AND PIG HEMOGLOBIN WERE SEPARATED FROM A HEMOLYZED MIXTURE OF HUMAN AND PIG BLOOD

Equal proportions of human and of pig blood were mixed and lysed, and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4A, pig hemoglobin separated virtually completely from human hemoglobin. This complete separation is surprising in light of the structural similarity between human and pig hemoglobin; pig and human alpha globin chains are 84.4 percent homologous and pig and human beta globin chains are 84.9 percent homologous. It is further surprising because, as shown in Figure 4C, when human and mouse blood was mixed, hemolyzed, applied to and eluted from a DEAE column according to methods set forth in Section 7.1.1., supra, human and mouse

hemoglobin were not observed to separate despite the fact that mouse and human alpha globin chains are about 85.8 percent homologous and mouse and human beta globin chains are 80.1 percent homologous. The ease of separation of human and pig hemoglobin on DEAE resin appears to be both efficient and economical.

Interestingly, the order of elution of the proteins from the anion exchange column was not as expected. Based on the relative pI's of the proteins as deduced from the IEF gels, the predicted order of elution would be first the hybrid (human α/pig β) followed by the authentic human α/human β. The last protein to elute from the anion exchange column then would be the endogenous pig α/pig β protein. However, under all the conditions currently attempted the order of elution was altered such that the human hemoglobin was the first to elute. The second peak was an enriched fraction of the hybrid followed very closely by the pig hemoglobin.

7.2.2. HUMAN AND PIG HEMOGLOBIN AND HUMAN/PIG HETEROLOGOUS HEMOGLOBIN WERE SEPARATED FROM HEMOLYSATE PREPARED FROM A TRANSGENIC PIG

Blood from transgenic pig 6-3 (as described in Section 6, supra) was lysed by hypotonic swelling and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4B, human hemoglobin was separated from pig hemoglobin and from human α globin/pig beta globin heterologous hemoglobin. As shown in Figure 4D, human hemoglobin was substantially purified by this method.

7.2.3. PIG ALPHA GLOBIN/HUMAN BETA GLOBIN HETEROLOGOUS HEMOGLOBIN DOES NOT APPEAR TO FORM BASED ON REASSOCIATION DATA

Heterologous association between pig alpha globin and human beta globin chains has not been detected in hemolysates obtained from human hemoglobin-expressing transgenic pigs. It was possible, however, that this observation could be explained by relatively low levels of human beta globin expression. Alternatively, association between pig alpha globin and human beta globin may be chemically unfavorable. In order to explore this possibility, reassociation experiments were performed in which pig and human hemoglobin were mixed, dissociated, and then the globin chains were allowed to reassociate. As shown in the isoelectric focusing gels depicted in Figure 5, although pig α/pig β, human α/human β, and human α/pig β association was observed, no association between pig α globin and human β globin appeared to have occurred. Therefore the pig α/human β heterologous hemoglobin should not be expected to complicate the purification of human hemoglobin from transgenic pigs.

8. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY QCPI CHROMATOGRAPHY

8.1. MATERIALS AND METHODS

Clarified hemolysate from transgenic pig 6-3 13mg/ml; Buffer A: 10mM Tris, 20mM Glycine pH 7.5; Buffer B: 10mM Tris, 20mM Glycine, 15 mM NaCl pH 7.5; Buffer C: 10mM Tris, 20mM Glycine, 1M NaCl pH 7.5; Buffer D: 10mM Tris, 20mM Glycine, 50 mM NaCl pH 7.5; QCPI column 10ml Equilibrated in Buffer A; Trio purification system. 10mg of hemoglobin prepared from transgenic pig 6-3 was diluted in 20ml Buffer A. 20ml of sample was loaded at a flow rate of 5ml/min onto the QCPI column, and washed with 2 column volumes of Buffer A. The column was then washed with 20 column volumes of a 0-50mM NaCl gradient. (10 column volumes

Buffer A + 10 column volumes of Buffer D) and the $O.D._{280}$ absorbing material was collected. The column was then cleaned with 2 column volumes of Buffer C, and then re-equilibrated with 2 column volumes of Buffer A.

8.2. RESULTS

Analysis of the UV trace (peak vs. volume of gradient) (Fig. 6) revealed that the human hemoglobin was eluted at 15 mM NaCl. Subsequent purifications have been performed utilizing the same protocol as above, only using 6 column volumes of Buffer B (15mM NaCl) to elute the human hemoglobin rather than the gradient. In addition, non-transgenic pig chromatographed by this method does not elute from the QCPI with Buffer B, while native human hemoglobin does. The protein that eluted at 15mM NaCl was analyzed on the Resolve isoelectric focussing system and found to be essentially pure of contaminating pig hemoglobin or hybrid hemoglobin.

9. EXAMPLE: HUMAN ALPHA/PIG BETA GLOBIN HYBRID HEMOGLOBIN EXHIBIT INCREASED P_{50}

As shown in Tables II and III, supra, transgenic pigs of the invention were all found to produce significant amounts of human α/pig β globin hybrid hemoglobin (the pig α/human β hybrid was not observed). Significantly, pigs that expressed higher percentages of hybrid also appeared to exhibit elevated P_{50} values for their whole blood (Figure 7).

10. EXAMPLE: ENHANCED EXPRESSION USING PIG BETA GLOBIN REGULATORY SEQUENCES

The 339 construct (Figures 1R and 12) containing the pig adult beta globin gene promoter region (Figure 8), was used to prepare transgenic pigs

according to the method set forth in Section 6.1.2. supra. Figure 15 depicts an isoelectric focusing gel analysis of hemoglobin produced by pig 70-3; equal amounts of hemoglobin from transgenic pig 6-3, carrying the 116 construct (Figure 1A) and human hemoglobin are run in adjacent lanes for comparison. As indicated by the brighter bands observed in the lane containing pig 70-3 hemoglobin at positions corresponding to human and hybrid hemoglobins (relative to the lane containing pig 6-3 hemoglobin), the amount of human hemoglobin produced by pig 70-3 is greater than the amount produced by pig 6-3. It has been calculated that 38 percent of the total hemoglobin produced by pig 70-3 is human hemoglobin, whereas 10 percent of total hemoglobin produced by pig 6-3 is human hemoglobin (see Table II and Section 6.2. supra, for data and calculations). This suggests that the pig beta globin promoter region is more efficient than the human beta globin promoter in transgenic pigs.

In a separate series of experiments, two more transgenic pigs, expressing human hemoglobin, were obtained using construct "339" (pigs 80-4 and 81-3) (FIG. 17). Human hemoglobin levels in these transgenic pigs was determined by running isoelectric focussing gels and densitometric scanning of the individual bands (FIG. 18). As indicated in Figure 17, both pig 70-3 and pig 80-4 expressed high levels of authentic human hemoglobin. To obtain the copy number of transgenes, genomic DNA (isolated from the tail) was digested with EcoR I and a Southern Blot was performed. The probe used was a 427 bp NcoI/Bam HI fragment of human beta globin gene containing the first exon, first intron and part of the second exon.

11. EXAMPLE: MOLECULAR MODELING OF PIG HEMOGLOBIN AND THE α_1 β_1 INTERFACE OF

A HYBRID BETWEEN PIG β AND HUMAN α GLOBIN

It has been found that the amount of hybrid human α/pig β hemoglobin often exceeds the amount of human hemoglobin. The molecular basis of this observation has been investigated using molecular modeling and molecular biology. The model structure of the hybrid molecule is based on the known structures of human hemoglobins and the structural homology between the human and pig structures (A.M. Lesk, 1991, Protein Architecture: A Practical Approach, Oxford University Press, N.Y.). The pig and hybrid hemoglobin structures were modeled using the following four steps: (1) hydrogen atoms were added to the X-ray model and their positions modified using energy minimization; (2) amino acid residue replacements were introduced to model the target pig and hybrid structures (no chain alignment was necessary); (3) the side chain positions of these modified residues were energy minimized; and (4) the result was visually examined and found to be sound. The modeled structures are shown in Figure 20.

Detailed examination of all the relevant contacts indicated striking differences at several residues. For example, at position β112 the human hemoglobin has a cysteine residue but the hybrid has a valine residue. The valine is in apparent closer contact (arrow in FIG. 20) with the opposing subunit, and thus may be more effective in stabilizing the α_1 β_1 interface (FIG. 21).

The effect of amino acid substitutions at the α_1 β_1 interface on the hydrophobic and polar interactions as predicted by HINT are shown in TABLE VI. HINT is software from Virginia Commonwealth University Licensed from Medical College of Virginia, Richmond, Virginia that can analyze the positive and negative scores as determined by attractive and

repulsive interactions known from experimental physical chemistry measurements. TABLE VI represents the differences between the unmodified dimer and the one with the specified replacement. TABLE VII has the same format as TABLE VI with the following two exceptions: (1) as each replacement is added, the previous one(s) are kept, and (2) the reported difference is a comparison between the current dimer and the one reflected in the preceding row. As the subsequential changes are made, the predicted attractive forces at the interface increase. If each column is summed up the total difference between the unmodified dimer and the one with seven changes is obtained. The sums are +1340 for hydrophobic and +660 for polar.

TABLE VI

Effect of amino acid replacements at the $\alpha 1\beta 1$ interface

Chain	Residue	Replacement	Predicted Difference	
			Hydrophobic	Polar
α	30	E to T	+250	+10
α	36	F to Y	-110	+220
α	106	L to F	+20	+10
α	107	V to S	-10	+120
α	107	V to C	0	+150
α	111	A to C	+30	+100
β	33	V to L	+70	0
β	112	C to V	+330	-60
β	112	C to I	+360	-50
β	115	A to V	+80	+10
β	115	A to L	+90	+10
β	119	G to H	+250	+120
β	125	P to M	+80	0
β	128	A to I	+80	0
β	131	Q to E	+120	+110

TABLE VII

Effect of combinations of amino acid replacements at the $\alpha 1\beta 1$ interface on the hydrophobic and polar interactions

Chain	Residue	Replacement	Predicted Difference	
			Hydrophobic	Polar
β	112	C to I	+360	-50
α	110	A to I	+200	+10
β	115	A to V	+150	+10
β	119	G to H	+270	+130
α	36	F to Y	-130	+240
β	33	V to L	+80	+0
α	30	E to T	+260	+10
β	131	Q to E	+150	+310

12. EXAMPLE: EXPRESSION OF GENETICALLY MODIFIED HEMOGLOBINS IN TRANSGENIC ANIMALS

Of the known human hemoglobin variants, about two dozen exhibit a lower oxygen affinity, which could be advantageous in clinical applications. While many of these mutants result in unstable hemoglobin molecules, several variants have desirable biochemical properties and can be used for the generation of blood substitutes using recombinant DNA technology. Transgenic pigs expressing two of these variants, Hb Presbyterian (108 Asn→Lys, Fig. 1G) and Hb Yoshizuka (108 Asn→Asp, Fig. 1F) have been produced and purification and characterization of the expressed human globins is described below.

12.1. PURIFICATION AND CHARACTERIZATION OF Hb PRESBYTERIAN

The amino acid substitution generated in Hb Presbyterian (β108 Asn→Lys) results in the comigration of Hb Presbyterian with the hybrid (hαpβ) hemoglobin on isoelectric focussing gels. Based on previous results with the purification of human hemoglobin from hybrid and porcine hemoglobins and the more positive nature of the Hb Presbyterian it should be easier to purify this variant hemoglobin on an anion exchange resin. Approximately 500 ml of blood was obtained from the transgenic pig 57-10. The blood was washed several times with isotonic saline and then lysed by hypotonic swelling in water. The cell membranes were removed by centrifugation at 10000 xg to yield a final hemoglobin concentration of about 100 mg/ml. Hb Presbyterian was purified from the hybrid and porcine hemoglobins as follows: 1-2.5 g of hemolysate was loaded onto an XK 50/30 column packed with 450 ml of Biorad Macroprep High Q resin equilibrated with 10 mM Tris-Cl and 20 mM Glycine at pH 8.1 (Buffer A). The

proteins were eluted at a flow rate of 10 ml/min with a linear salt gradient of 9-16% Buffer B (Buffer A containing 250 mM NaCl) over 3000 ml.

The initial peak was thought to be Hb Presbyterian followed by the co-elution of the hybrid and porcine hemoglobins (FIG. 20). To confirm the identity of the first peak as Hb Presbyterian and not the hybrid hemoglobin, a sample of the protein was run on Reversed Phase HPLC (FIG. 21). The initial peak from the anion exchange column was Hb Presbyterian with the α-chains eluting at the same time as normal human α-chains and the β-chains eluting slightly faster than normal human β-chains. This was also found to be an excellent way of determining if porcine hemoglobin was contaminating the column fractions. Using this purification procedure and the analysis on HPLC the recombinant Hb Presbyterian derived from the transgenic pig 58-10 was judged to be greater than 95% pure.

Purified Hb Presbyterian was dialyzed against 50 mM HEPES and 100 mM NaCl at pH 7.4 and oxygen equilibrium curves determined using a Hemox Analyzer (TCS Products, Southampton, PA). The Hemox Analyzer was modified to allow analog to digital data conversion for ease of oxygen binding calculations. Under these conditions the Hb Presbyterian had a P_{50} of 25.8 mmHg (Hill Coefficient n=2.3) versus 13.3 mm Hg (n=2.9) for Hb A indicating that the Hb Presbyterian bound oxygen with lower affinity than native Hb. Preliminary results to determine the Bohr Effect (Influence of pH on the oxygen affinity) indicated a normal Bohr effect for Hb Presbyterian (FIG. 22).

12.2. PURIFICATION AND CHARACTERIZATION OF Hb YOSHIZUKA

Blood samples taken from the transgenic pigs expressing Hb Yoshizuka (68-3 and 68-2) were treated essentially the same as described above. The final concentration of the hemolysate was approximately 100 mg/ml. The purification of the protein required a slightly different strategy, however. A sample of hemolysate from 68-3 (about 10 mg) was loaded onto an HR 10/30 Biorad Macroprep High Q resin column equilibrated with 10 mM Tris-Cl and 20 mM Glycine at pH 8.7 (Buffer A). The hemoglobins were eluted at 2.5 mls/min with a 5-30% linear gradient of Buffer B (Buffer A plus 250 mM NaCl) over 500 ml (FIG. 23). Fractions were collected and analyzed by IEF to assess purity which was determined to be about 75% or better.

13. DEPOSIT OF MICROORGANISMS

The following plasmids were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 on December 2, 1992.

plasmid	containing	accession no.
psaf/pigε(k)	pigε globin gene	75371
pGem5/Pigβpr(K)	pig adult β globin gene regulatory region	75372
pPig3'β	3' end of pig β globin gene	75373

Various publications are cited herein which are hereby incorporated by reference in their entirety.

International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 52, lines 18-28 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet.

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, MD 10582
US

Date of deposit December 2, 1992 Accession Number 75371

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☒

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)

Allison L. Brinkfield
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 10582
US

Accession No.	Date of Deposit
75372	December 2, 1992
75373	December 2, 1992

WHAT IS CLAIMED IS:

1. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid construct is the 426 construct as depicted in Figure 14.

2. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid construct is the 427 construct as depicted in Figure 14.

3. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the amount of human globin produced relative to total hemoglobin is at least twenty percent.

4. A transgenic pig comprised of a DNA sequence comprising the pig adult β globin regulatory region as contained in plasmid pGem5/Pigβpr(K), deposited with the American Type Culture Collection and assigned accession number 75371, operably linked to a gene, in which the gene does not encode pig adult β globin, where the gene is expressed in at least some of the red blood cells of said pig.

5. The transgenic pig of claim 4 in which the gene is human β globin.

6. The transgenic pig of claim 4 in which the gene encodes a non-globin protein.

7. A transgenic pig comprised of a DNA sequence comprising the 3' region of the pig adult β globin gene, as contained in plasmid pPig3'β, deposited with the American Type Culture Collection and assigned accession number 75372, operably linked to a gene, in which the gene is not pig adult β globin, where the gene is expressed in at least some of the red blood cells of said pig.

8. The transgenic pig of claim 7 in which the gene is human β globin.

9. The transgenic pig of claim 7 in which the gene encodes a non-globin protein.

10. A purified and isolated nucleic acid comprising: the pig adult β globin regulatory region as comprised in plasmid pGem5/Pigβpr(K), as deposited with the American Type Culture Collection and assigned accession number 75371.

11. A purified and isolated nucleic acid comprising: the pig ε globin gene as comprised in plasmid pSaf/pigε(K), as deposited with the American Type Culture Collection and assigned accession number 75373.

12. A purified and isolated nucleic acid comprising: the 3' region of the pig adult β globin gene as comprised in plasmid pPig3'β, as deposited with the American Type Culture Collection and assigned accession number 75372.

13. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid encoding human alpha globin or human beta globin comprises a mutation which increases the level of authentic human/human dimer in the transgenic pig.

14. The transgenic pig of claim 13 wherein the mutation in human alpha hemoglobin is selected from the following group of alpha-chain mutations: a Thr at position 30 instead of Glu; a Tyr at position 36 instead of Phe; a Phe instead of Leu at position 106; a Ser or Cys instead of Val at position 107; and a Cys instead of Ala at position 111.

15. The transgenic pig of claim 13 wherein the mutation in human beta hemoglobin is selected from the following group of beta-chain mutations: a Leu instead of Val at position 33; a Ile instead of Cys at position 112; a Val or Leu instead of Ala at position 115; a His instead of Gly at position 119; a Met instead of Pro at position 128; and a Glu instead of Gln at position 131.

16. The transgenic pig of claim 15 wherein the mutation in human beta hemoglobin is a Cys to Val change at position 112.

17. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid construct is the hemoglobin Presbyterian construct as depicted in Figure 1G.

18. A method for purifying human Presbyterian Hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

(i) collecting red blood cells from a transgenic pig according to claim 17;

(ii) releasing the contents of the collected red blood cells to produce a lysate;

(iii) applying the lysate of step (ii) to a High Q resin column equilibrated with 20 mM Tris-Cl and 20 mM Glycine at a pH 8.1;

(iv) eluting the column with a linear salt gradient of 9-16% in buffer containing 10mM Tris-Cl, 20mM Glycine, 250mM NaCl at pH 8.1; and

(v) collecting the fractions that contain purified human Presbyterian Hb.

19. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and The transgenic pig of claim 1 in which the nucleic acid construct is the hemoglobin Yoshizuka construct as depicted in Figure 1F.

20. A method for purifying human Yoshizuka Hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

(i) collecting red blood cells from a transgenic pig according to claim 19;

(ii) releasing the contents of the collected red blood cells to produce a lysate;

(iii) applying the lysate of step (ii) to a High Q resin column equilibrated with 10mM Tris-Cl and 20mM Glycine at a pH 8.7;

(iv) eluting the column with a linear salt gradient of 5-30% in buffer containing 10mM Tris-Cl, 20mM Glycine, 250mM NaCl at pH 8.7; and

(v) collecting the fractions that contain purified human Yoshizuka Hb.

αβ

CONSTRUCT #116

(16.9 kb)

FIG. 1A

α-Promoter-β

CONSTRUCT #185

(13.5 kb)

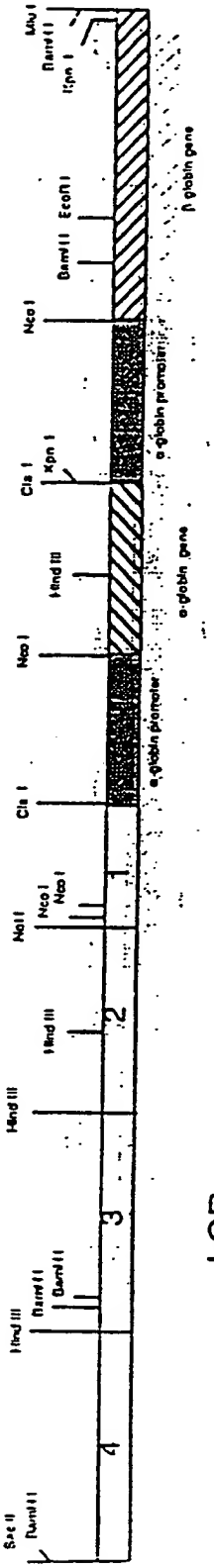


FIG. 18

β-Promoter-α
CONSTRUCT #290
(13.9 kb)

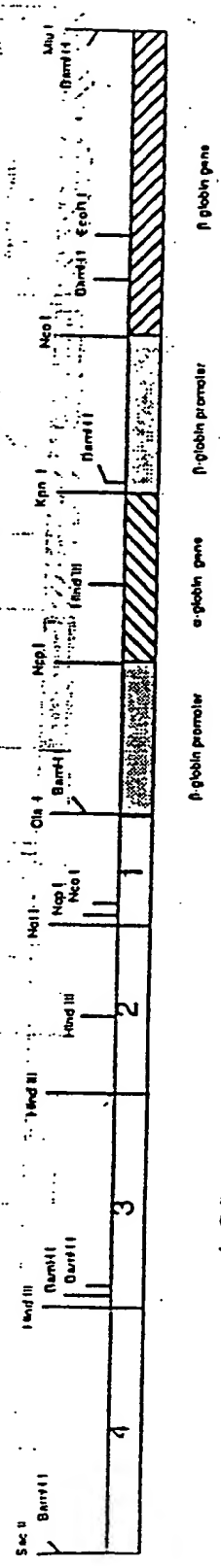


FIG. 1C

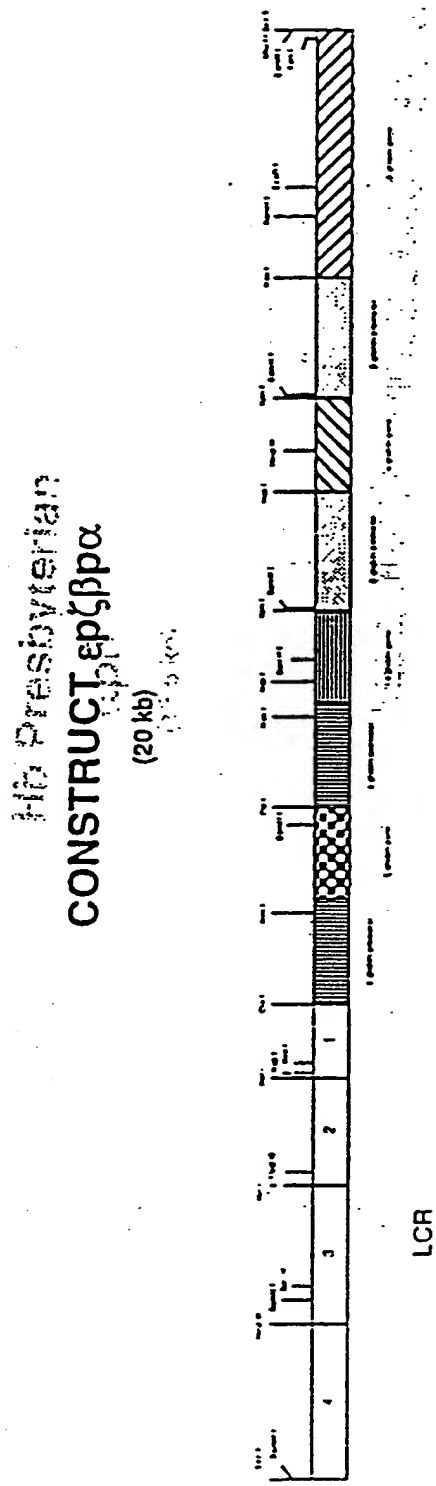


FIG. 1D

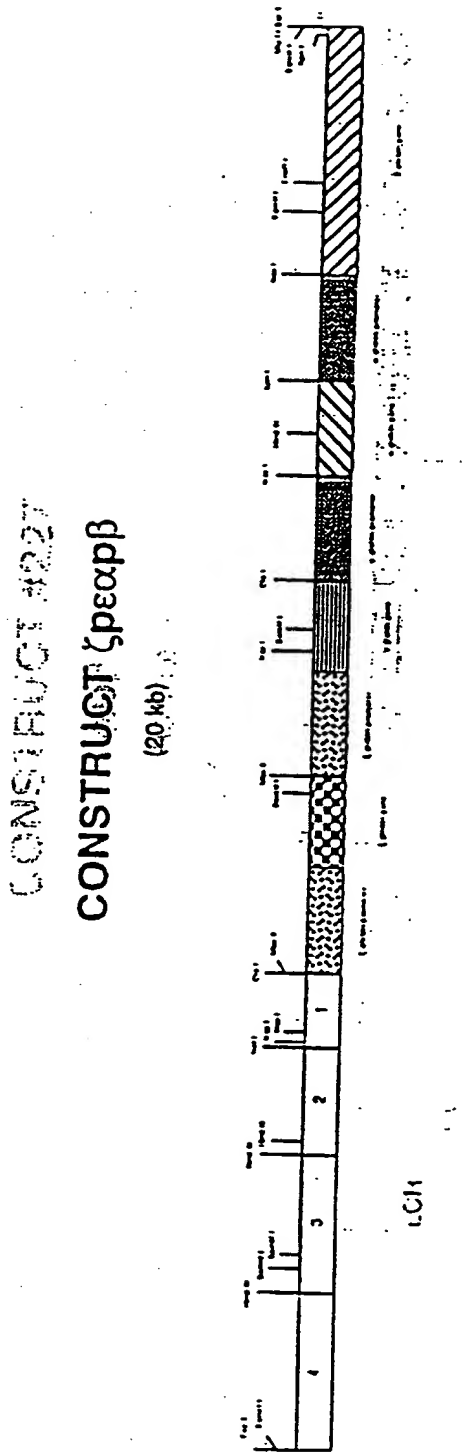


FIG. 1E

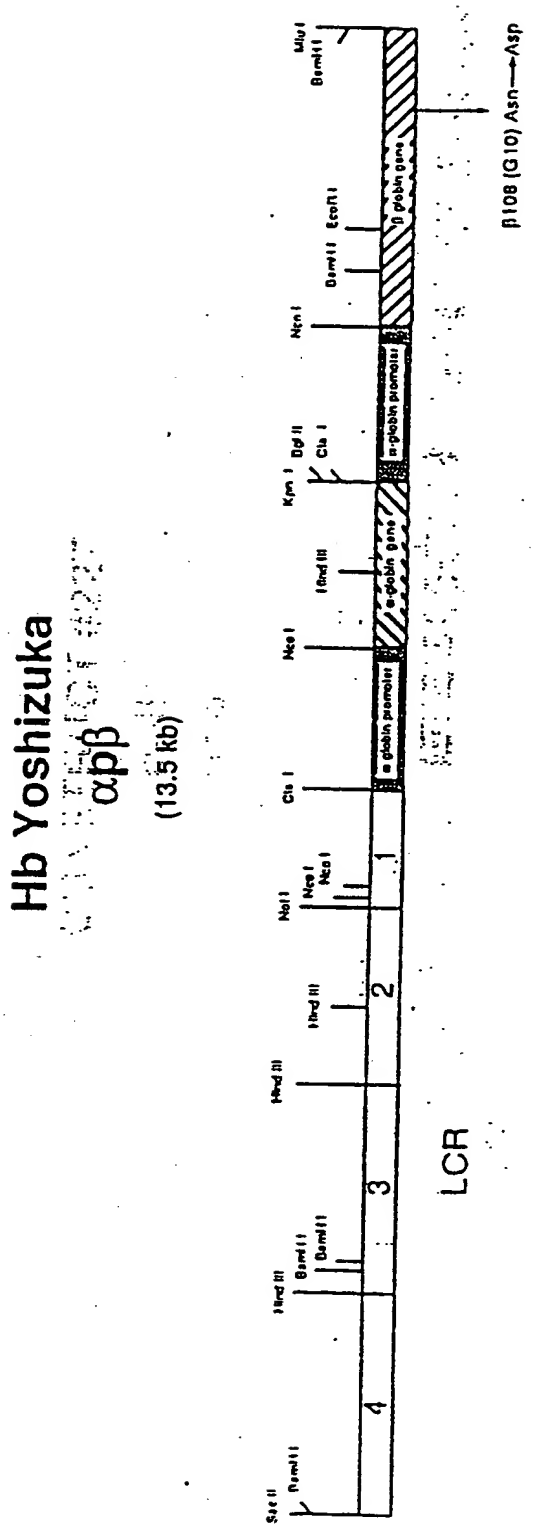

Hb Yoshizuka
αpβ
(13.5 kb)
LCR
β108 (G10) Asn→Asp
1
2
3
4
Nat I
Nco I
Cla I
Hind III
Bam H I
Eco R I
Kpn I
Bgl II
Sac II

FIG. 1F

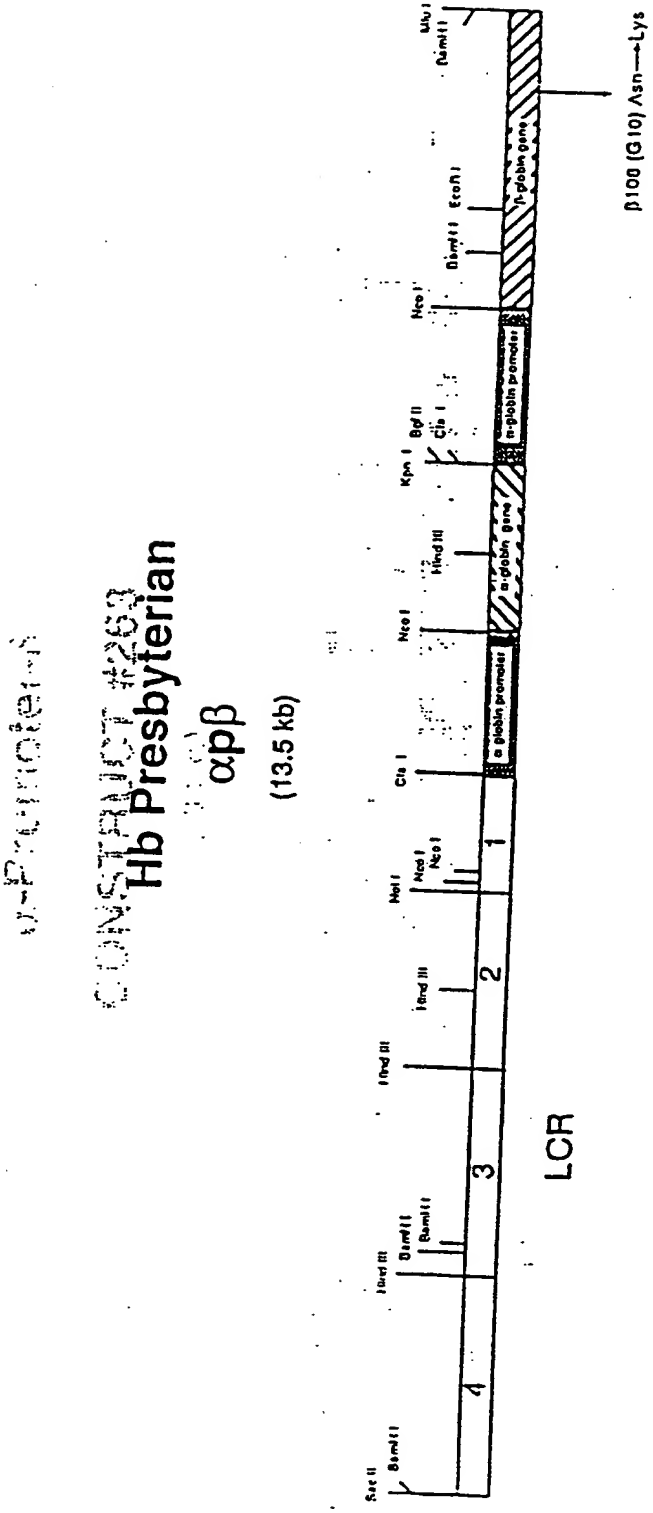
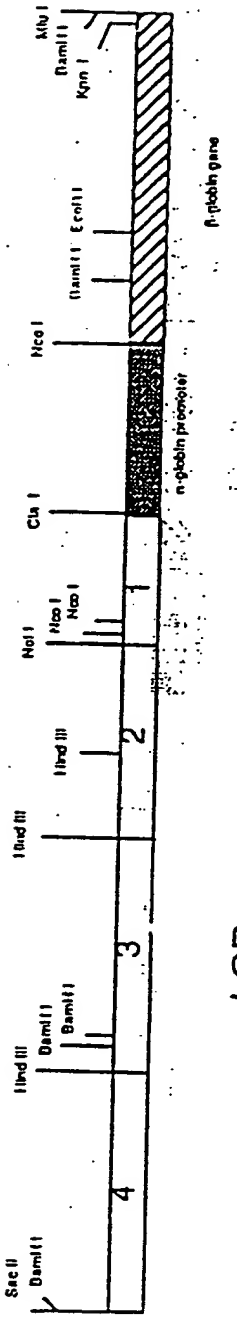


FIG. 1G

CONSTRUCT #285

α-Promoter-β (Δα)

(10.8 kb)



LCR α

(9.2 kb)

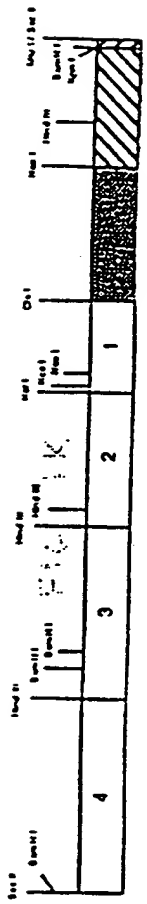


FIG 1 H

CONSTRUCT #227

αpβ

(13.5 kb)

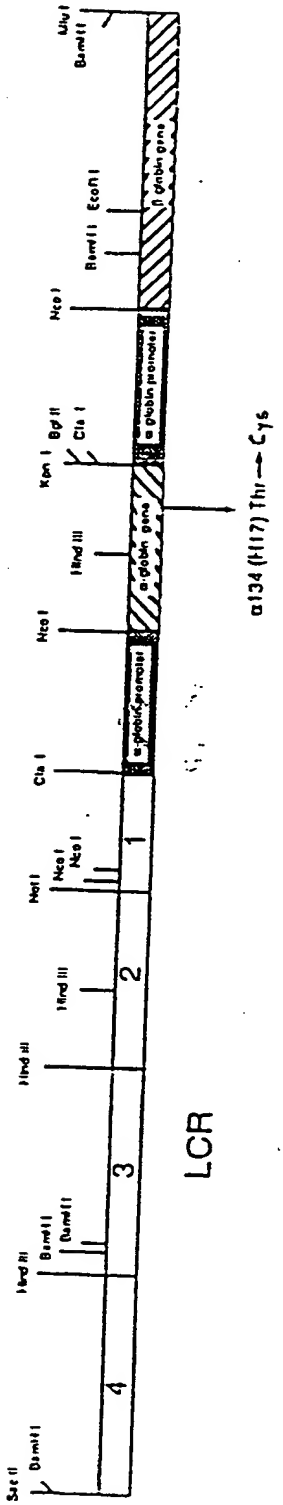


FIG. 11

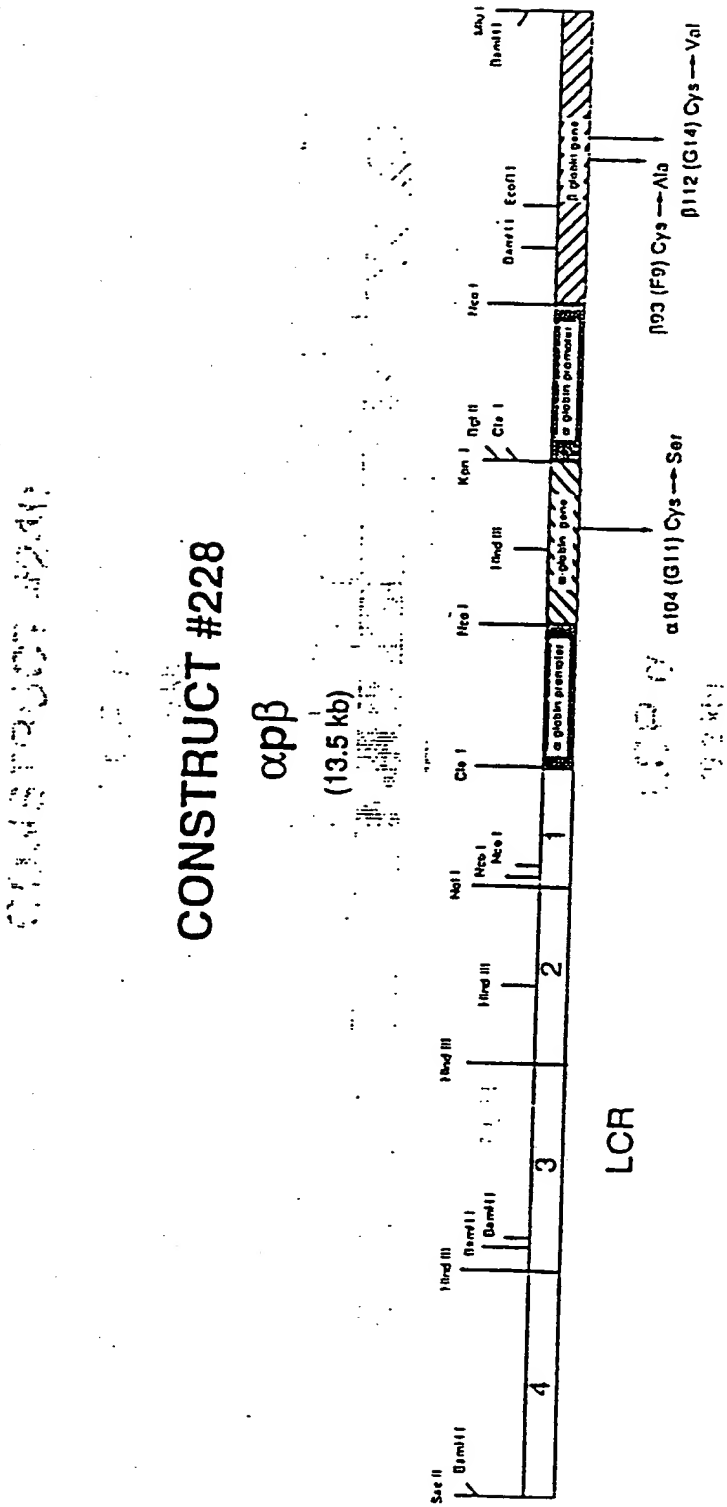

CONSTRUCT #228
αpβ
(13.5 kb)
LCR
1
2
3
4
α104 (G11) Cys → Ser
β93 (F9) Cys → Ala
β112 (G14) Cys → Val

FIG 1J

α-Promoter-δ

CONSTRUCT #263

(13.1 kb)

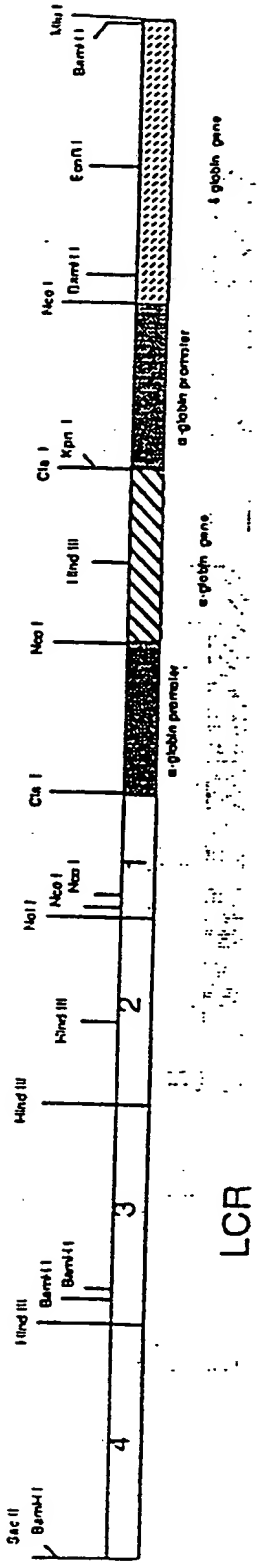


FIG. 1K

CONSTRUCT #274

α-Promoter-δ (Δα)

(10.4 kb)

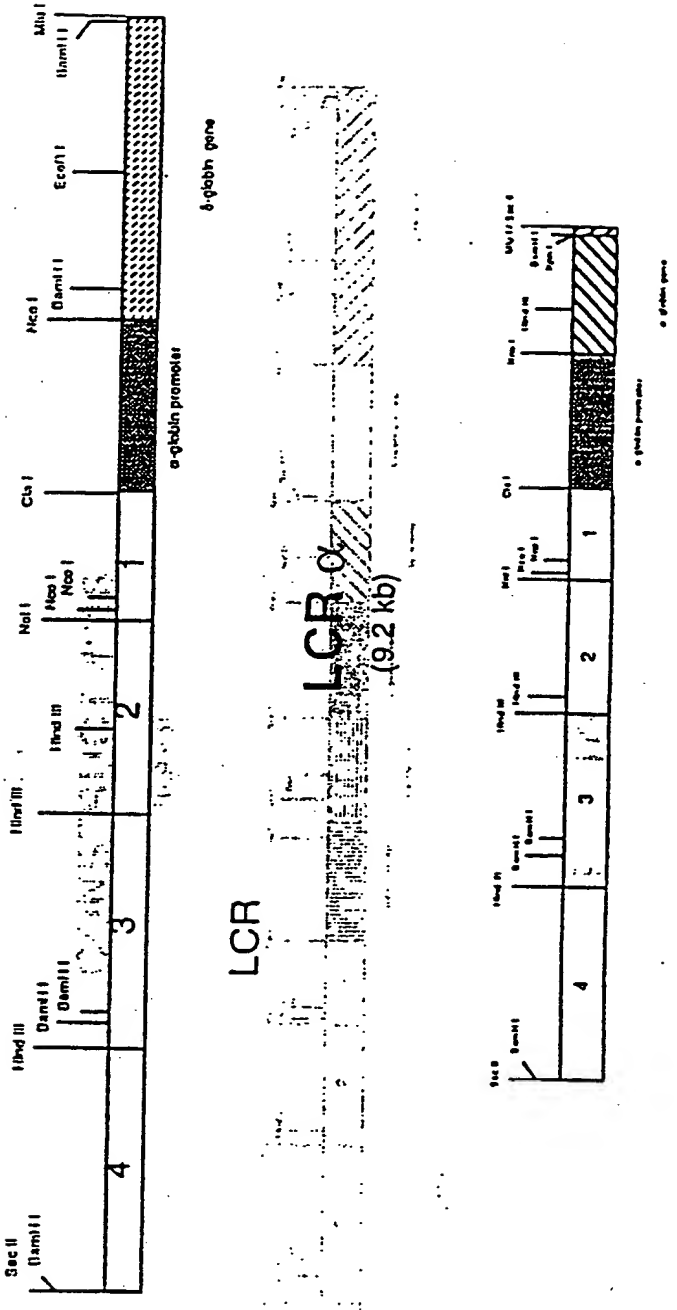
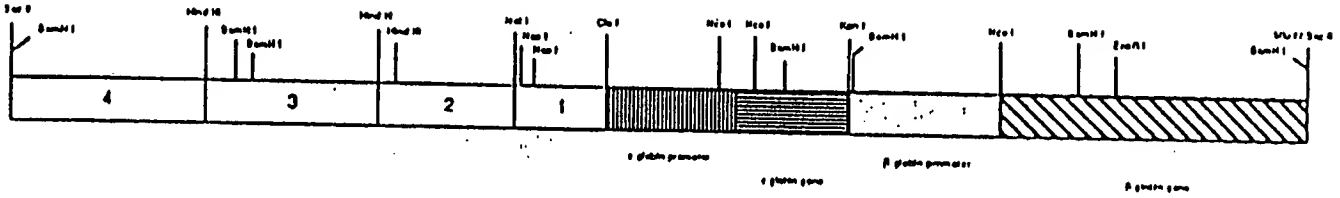


FIG. 1L

CONSTRUCT #240

LCR εβ

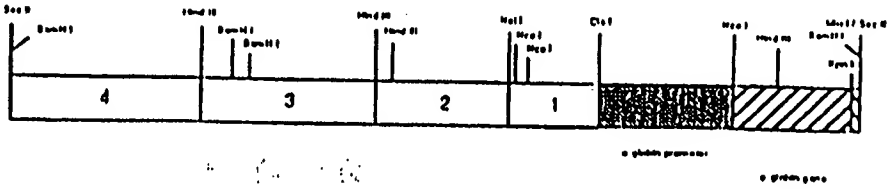
(14.0 kb)



LCR

LCR α

(9.2 kb)



LCR

FIG. 1M

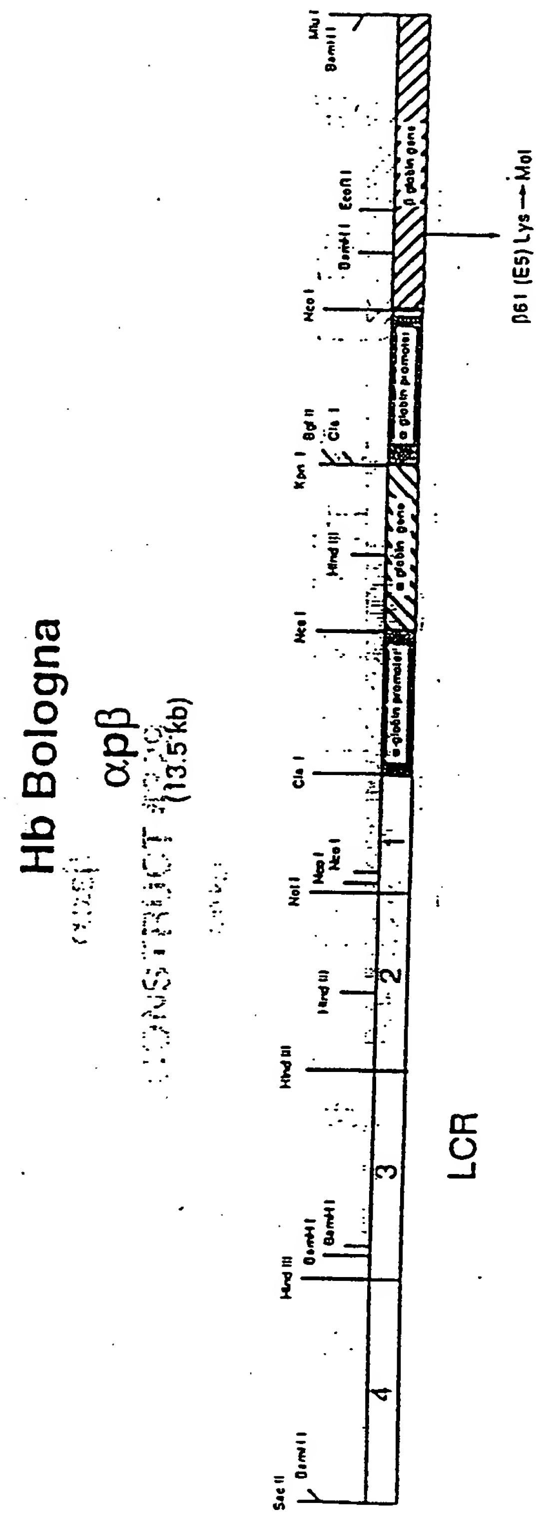

Hb Bologna
αpβ
(13.5 kb)
LCR
4
3
2
1
β61 (E5) Lys → Met
α globin promoter
α globin gene
β globin gene

FIG. 1N

εαβ

CONSTRUCT #318

(16.9kb)

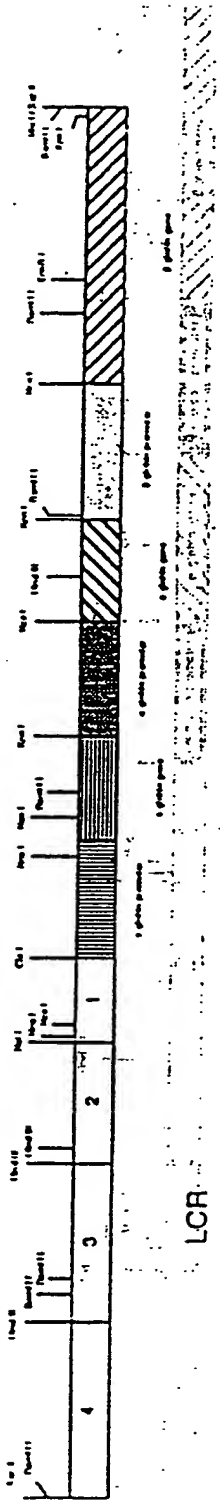


FIG. 10

αεβ

CONSTRUCT #319

(16.9kb)

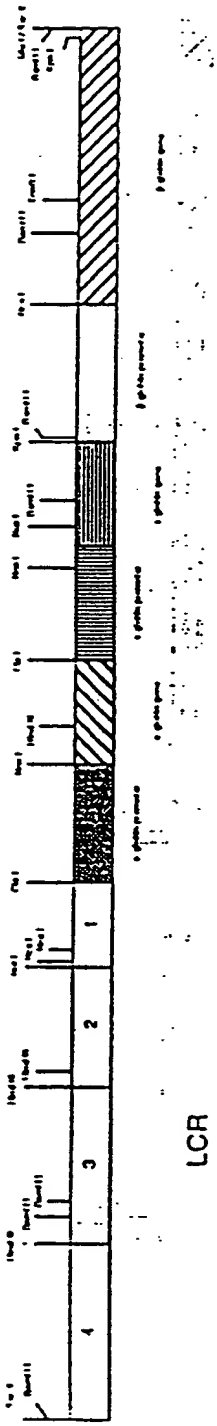


FIG. 1P

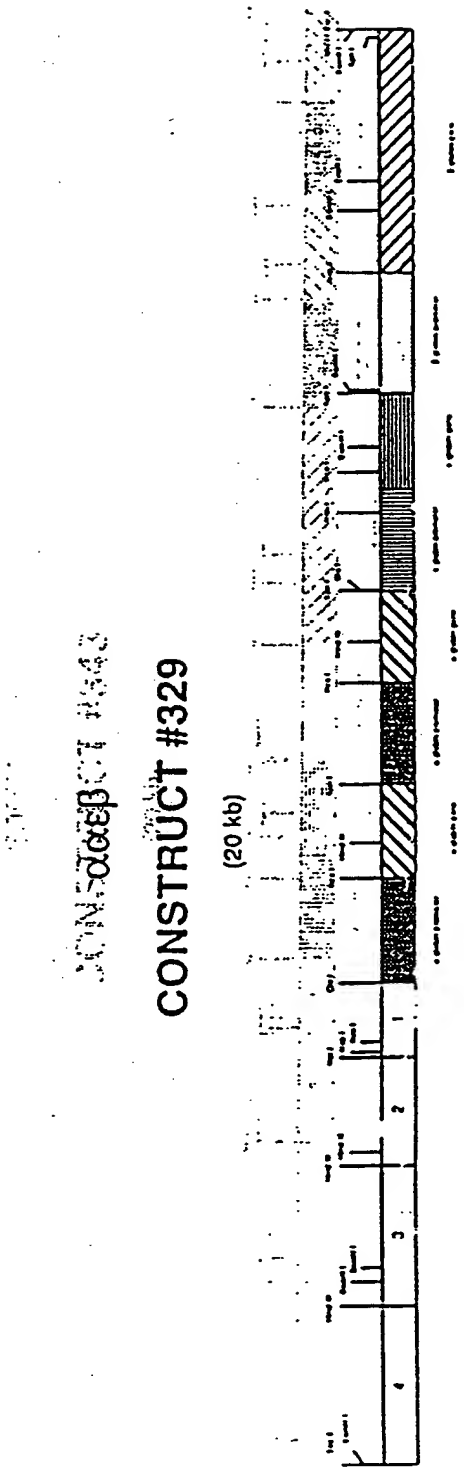


FIG 1Q

αε(globin)pβ

CONSTRUCT #339

(18 kb)

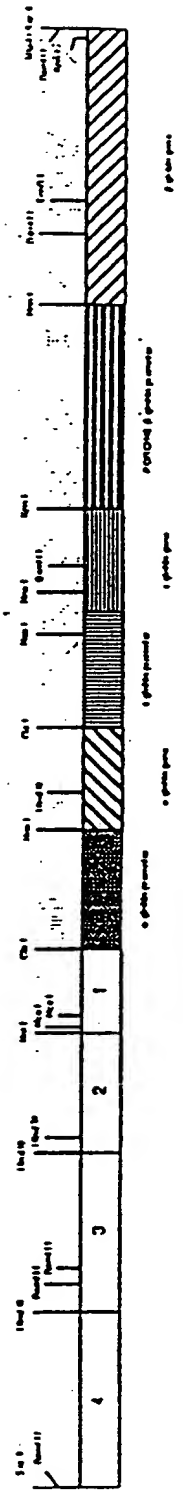


FIG. 1R

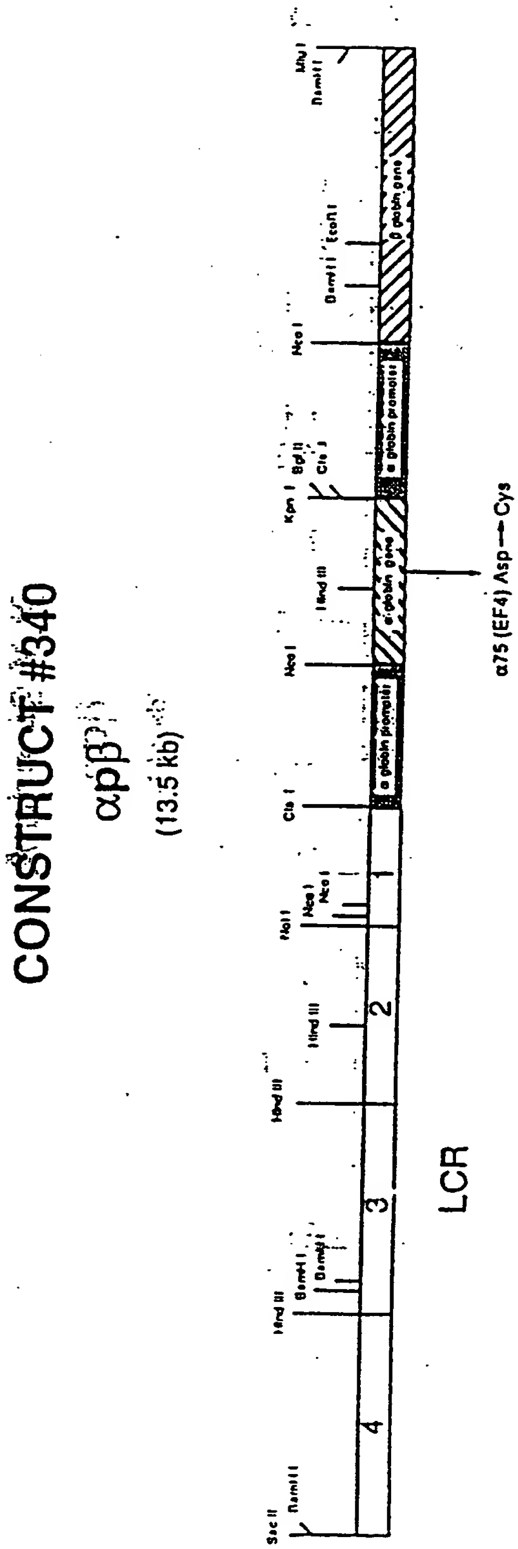

CONSTRUCT #340
αpβ
(13.5 kb)
LCR
4
3
2
1
α globin promoter
α globin gene
β globin gene
α75 (EF4) Asp→Cys
Sac II
BamH I
Hind III
Nco I
Cla I
Kpn I
Bgl II
EcoR I
Mlu I

FIG. 15

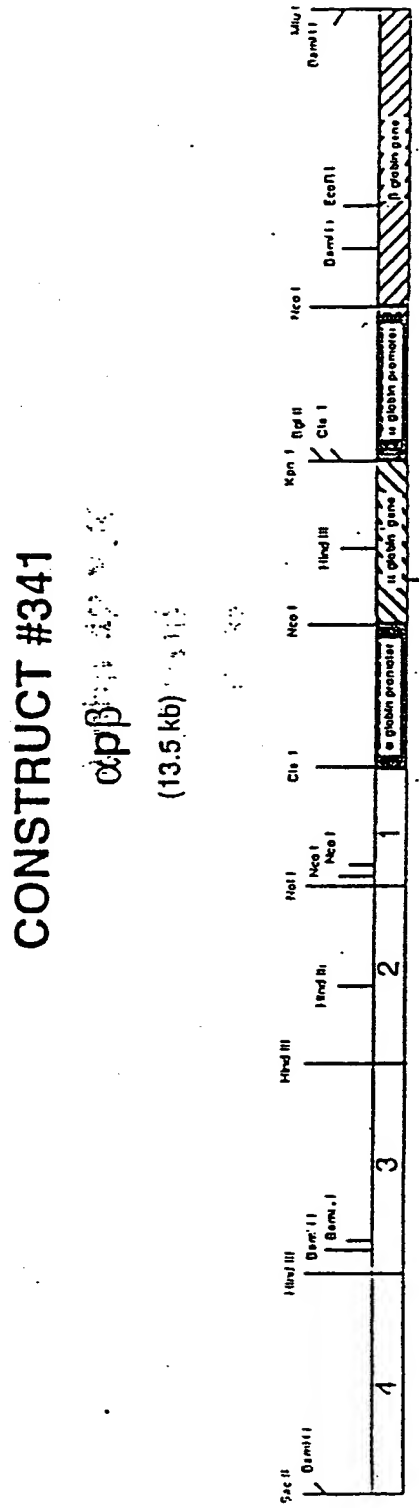

CONSTRUCT #341
αpβ
(13.5 kb)
LCR
1
2
3
4
α42 (C7) Tyr→Arg
Sac II
BamH I
Hind III
Nco I
Cla I
Kpn I
Bgl II
Xba I
EcoR I

FIG. 1T

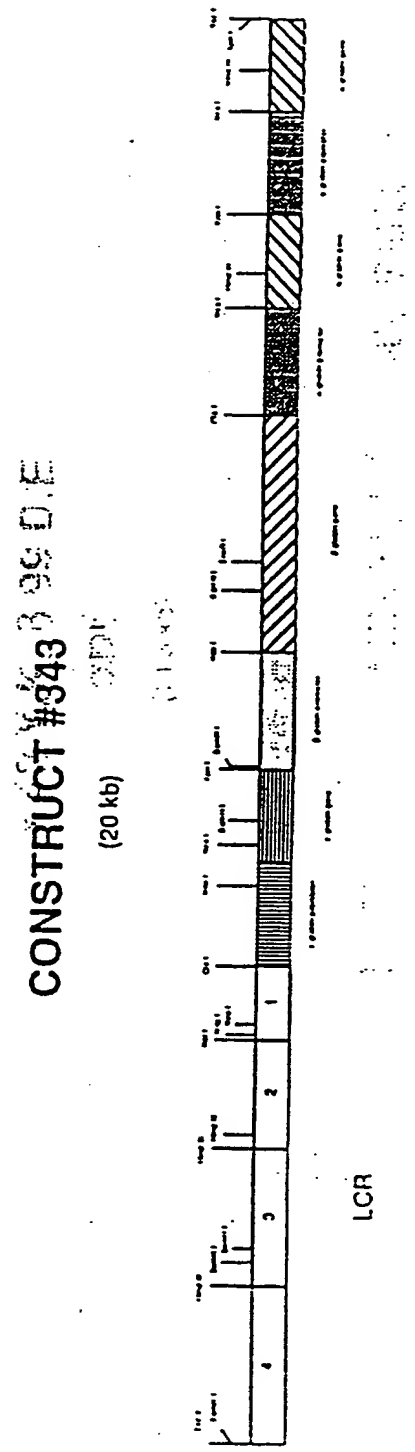


FIG. 11

εβα

CONSTRUCT #347

(16.9 kb)

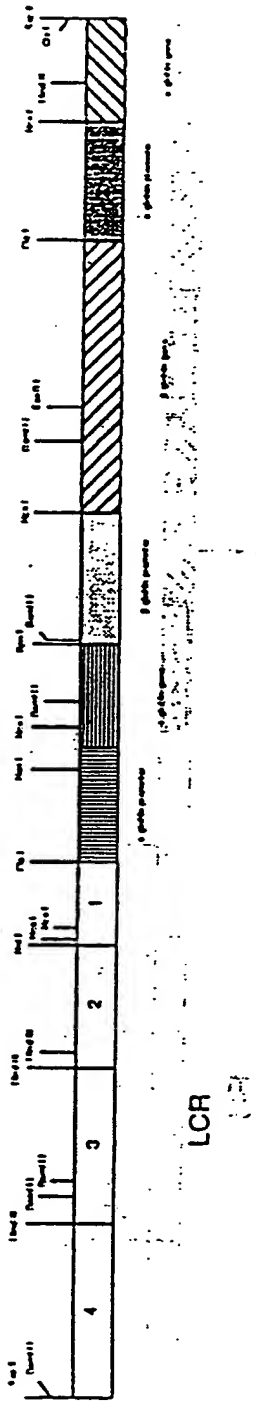


FIG. IV

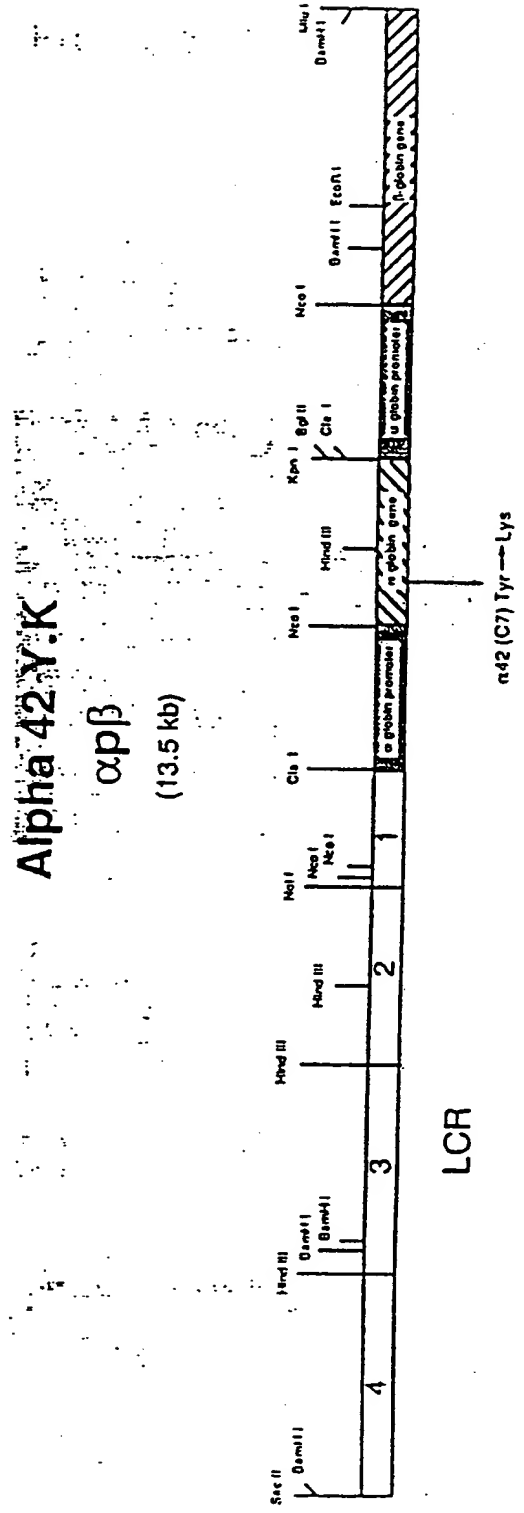

Alpha 42 Y:K
αpβ
(13.5 kb)
α42 (C7) Tyr—Lys
LCR
1
2
3
4
ζ globin gene
ζ globin promoter
α globin gene
α globin promoter
μ globin gene
μ globin promoter
Bam H I
Eco R I
Nco I
Bgl II
Cla I
Kpn I
Hind III
Not I
Sac II

FIG. 1W

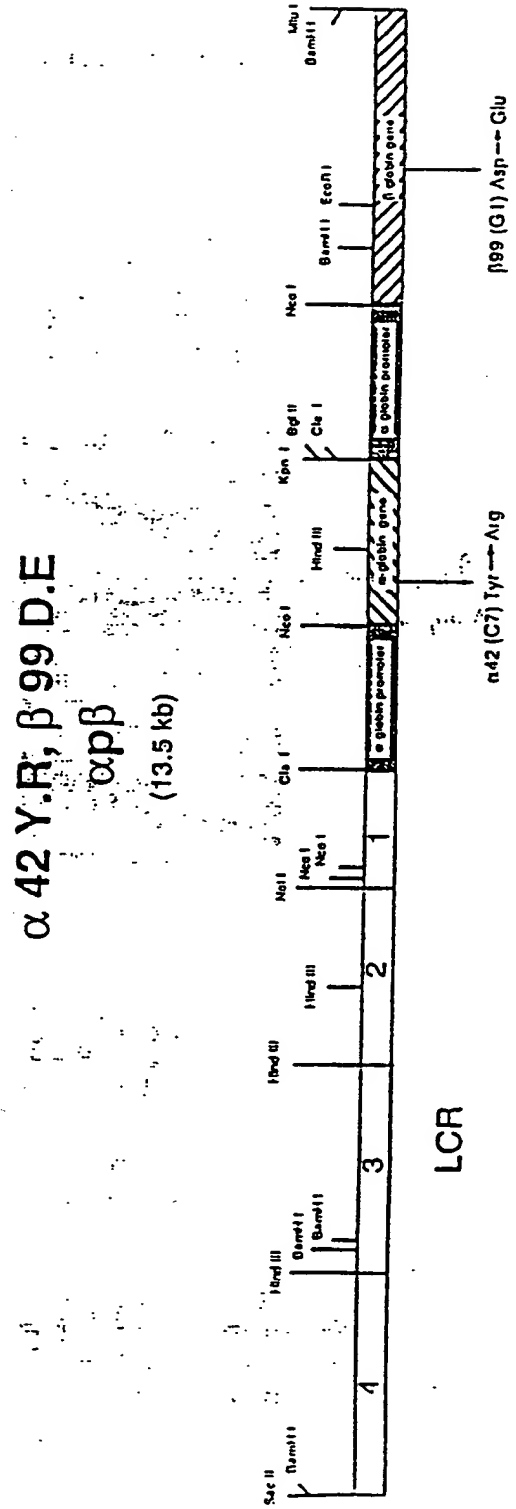


FIG. 1X

α 42 Y.K, β 99 D.E

αpβ

(13.5 kb)

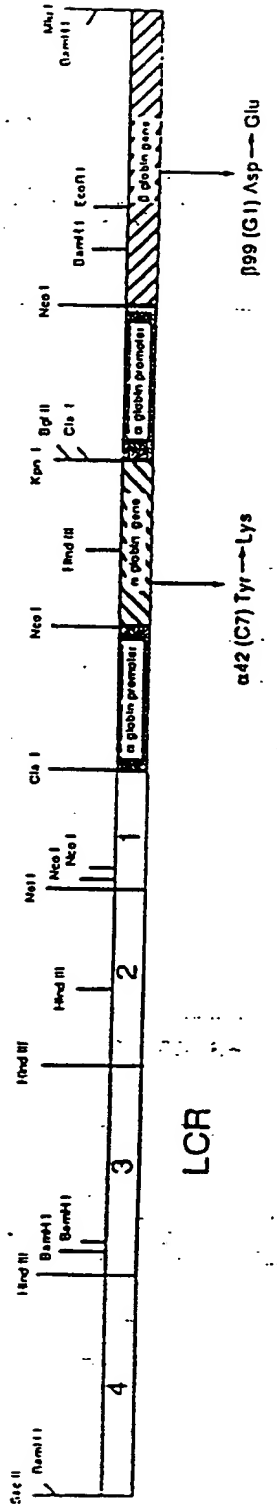


FIG. 1Y

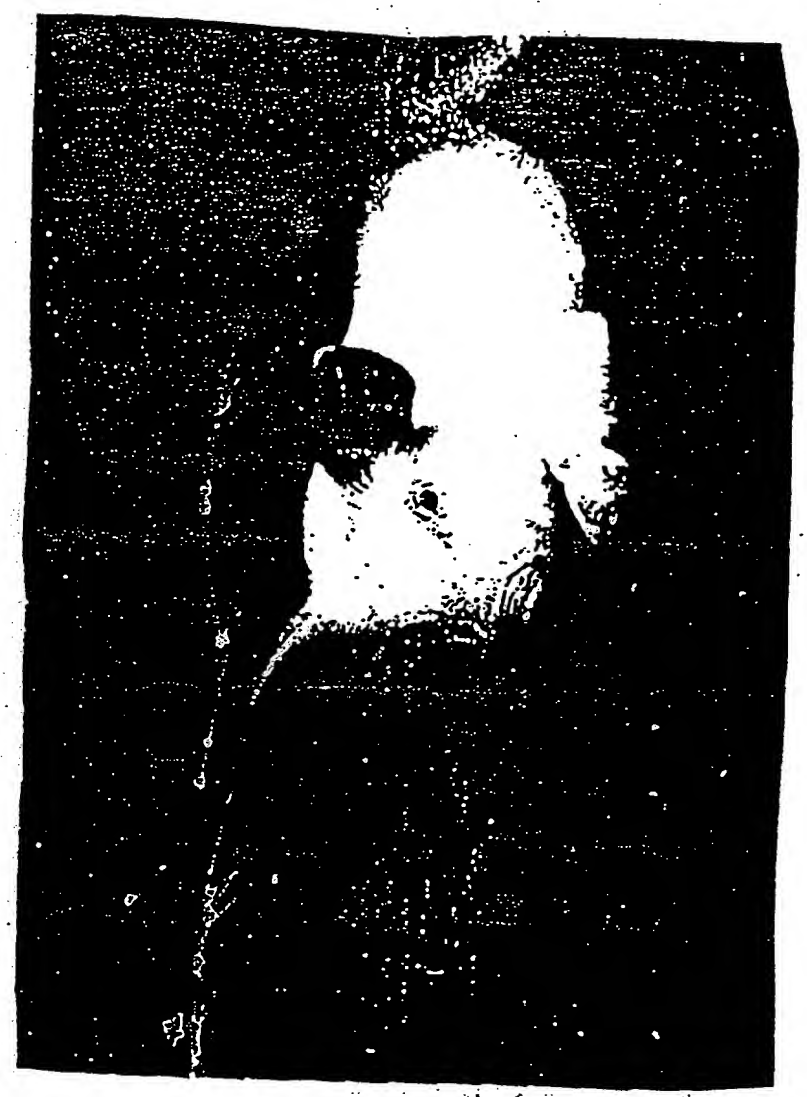
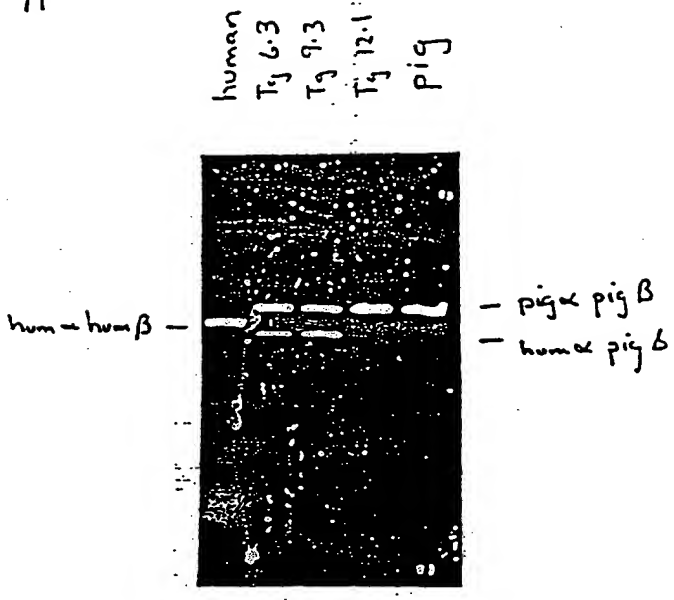


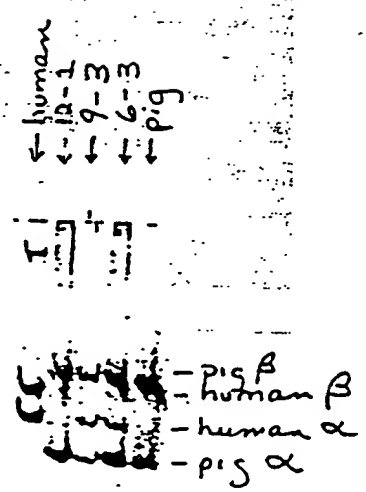
FIG. 2

FIG. 3 A-B

A


human
Tg 6.3
Tg 9.3
Tg 12.1
pig
hum α hum β
pig α pig β
hum α pig β

B.


human
12-1
9-3
6-3
pig
pig β
human β
human α
pig α

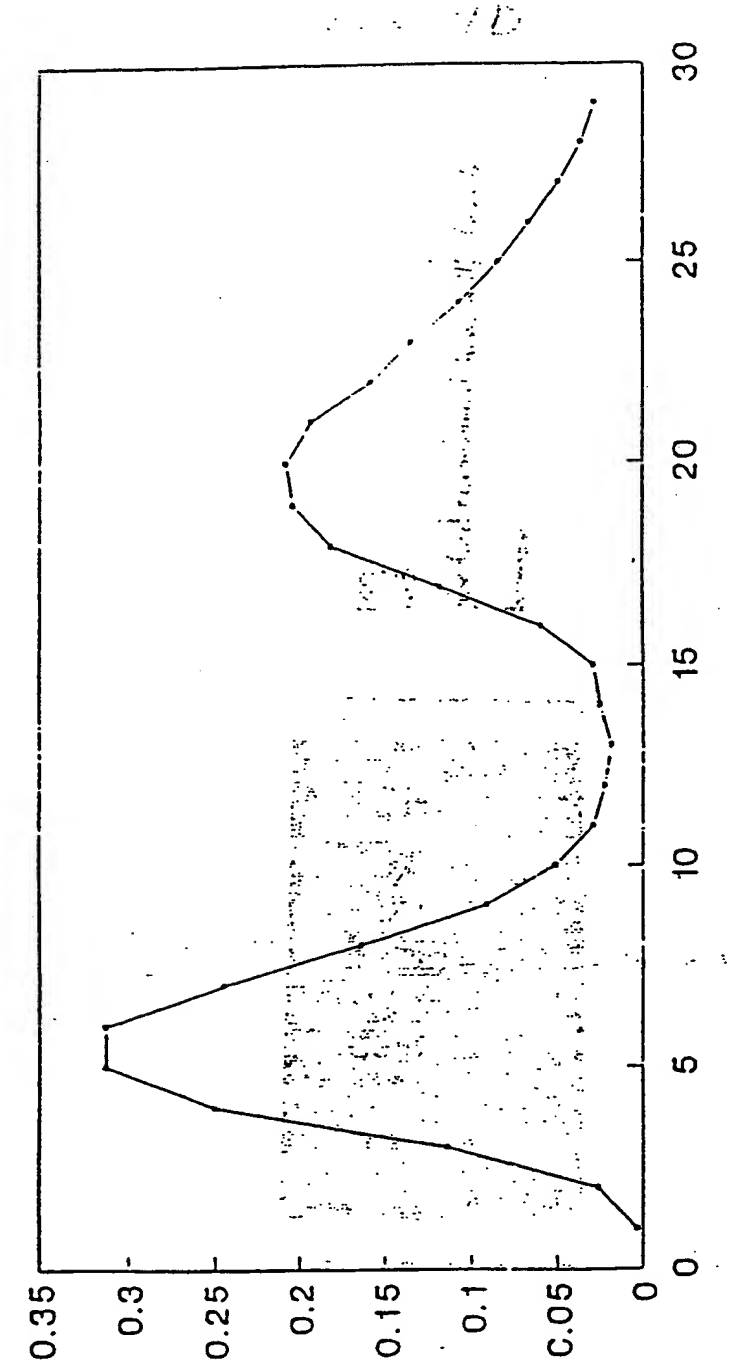
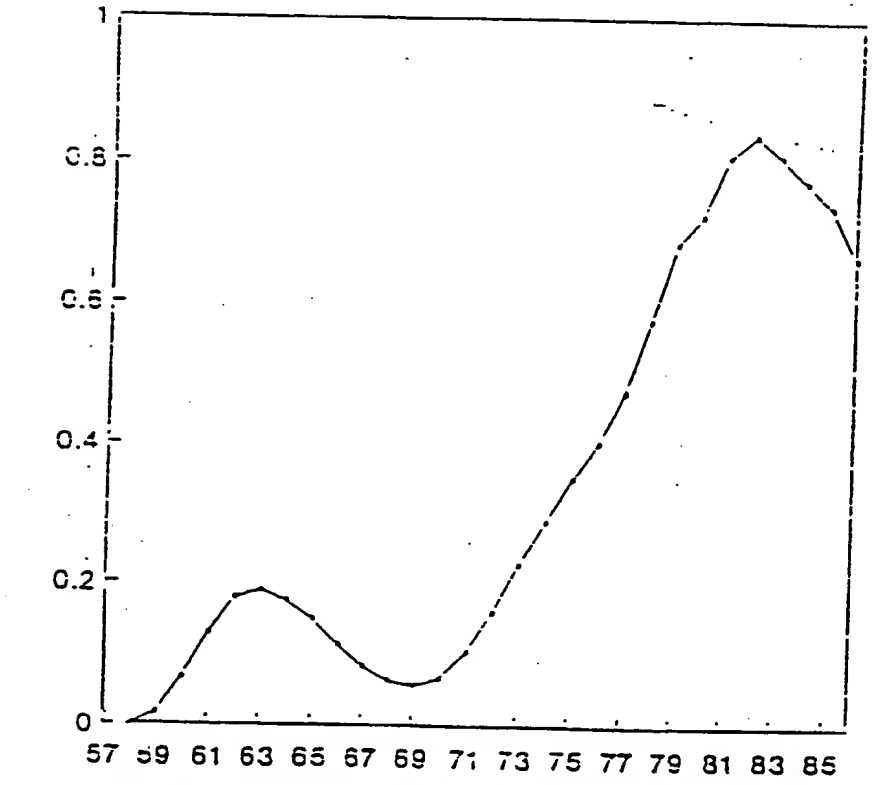

50:50 Pig/Human Mix
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
1:10 dilutions (129-2)

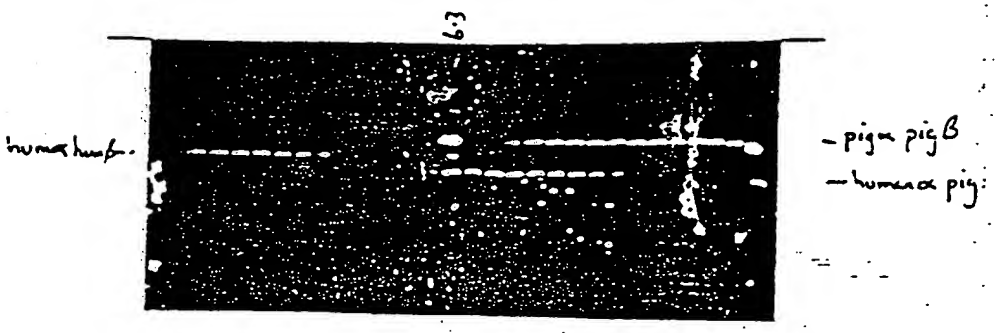
FIG. 4A

FIG. 4B

Pig 6-3

5 to 30 mM NaCl grad.





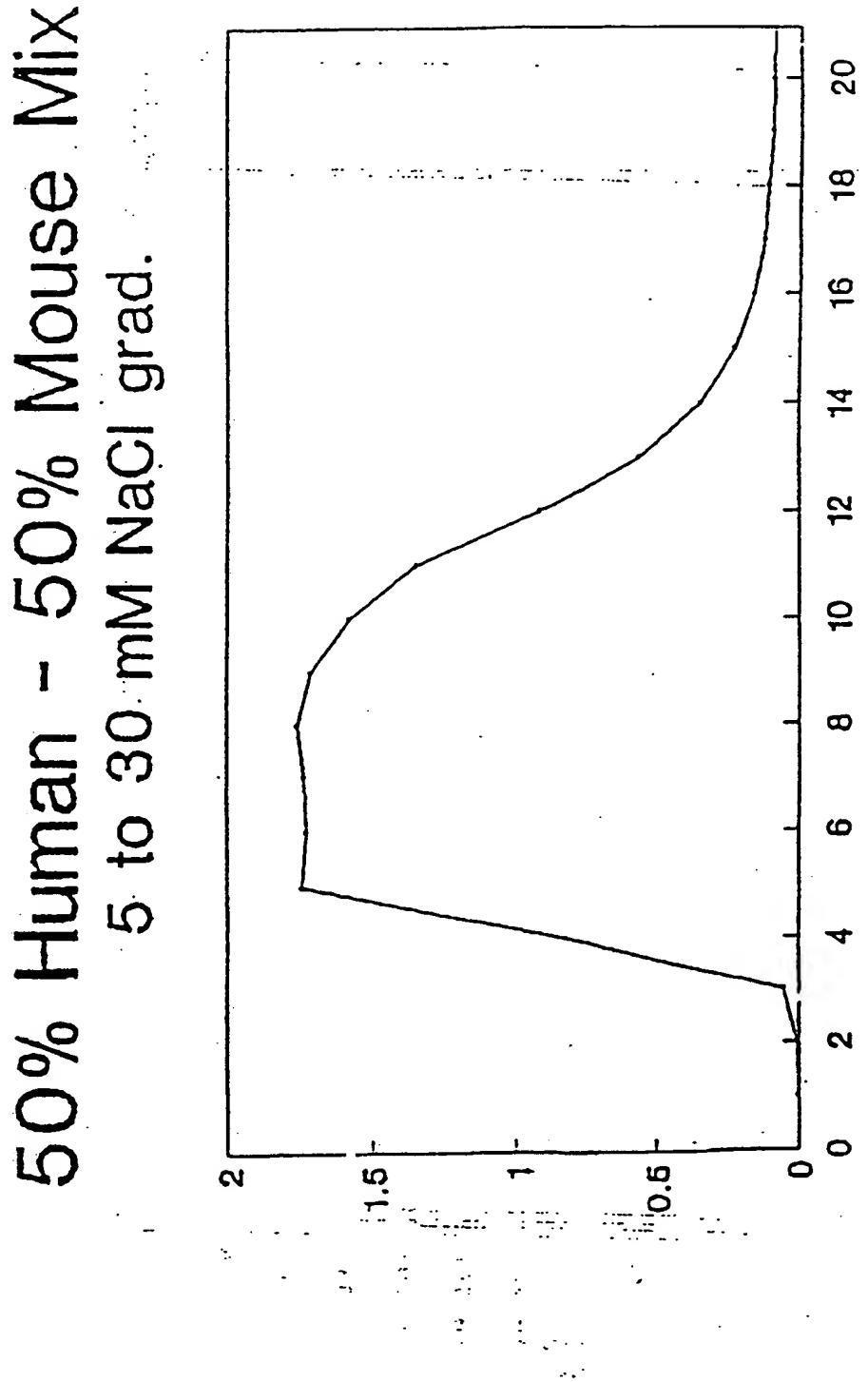

50% Human - 50% Mouse Mix
5 to 30 mM NaCl grad.
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
20
Fraction

FIG. 4C

FIG. 4D

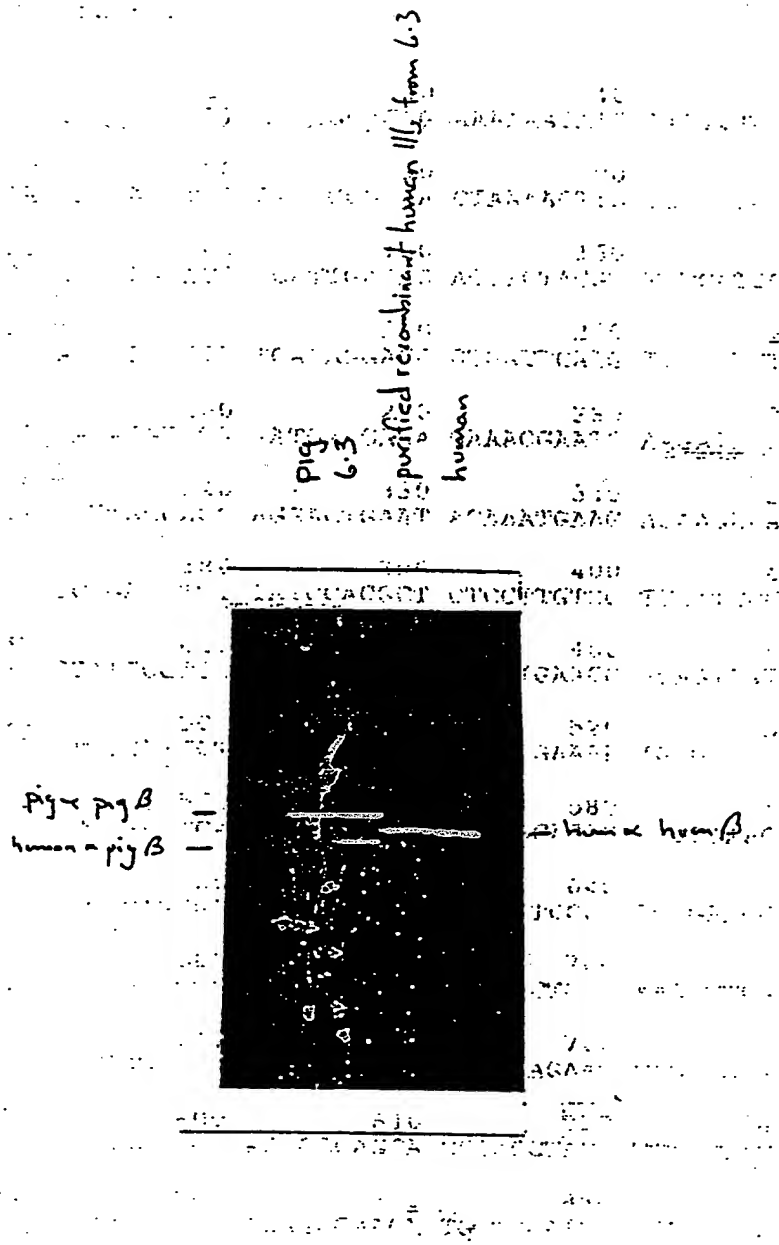

Pig 6.3
purified recombinant human IIIa from 6.3
human
pig α pig β
human - pig β
human α human β

FIG. 5

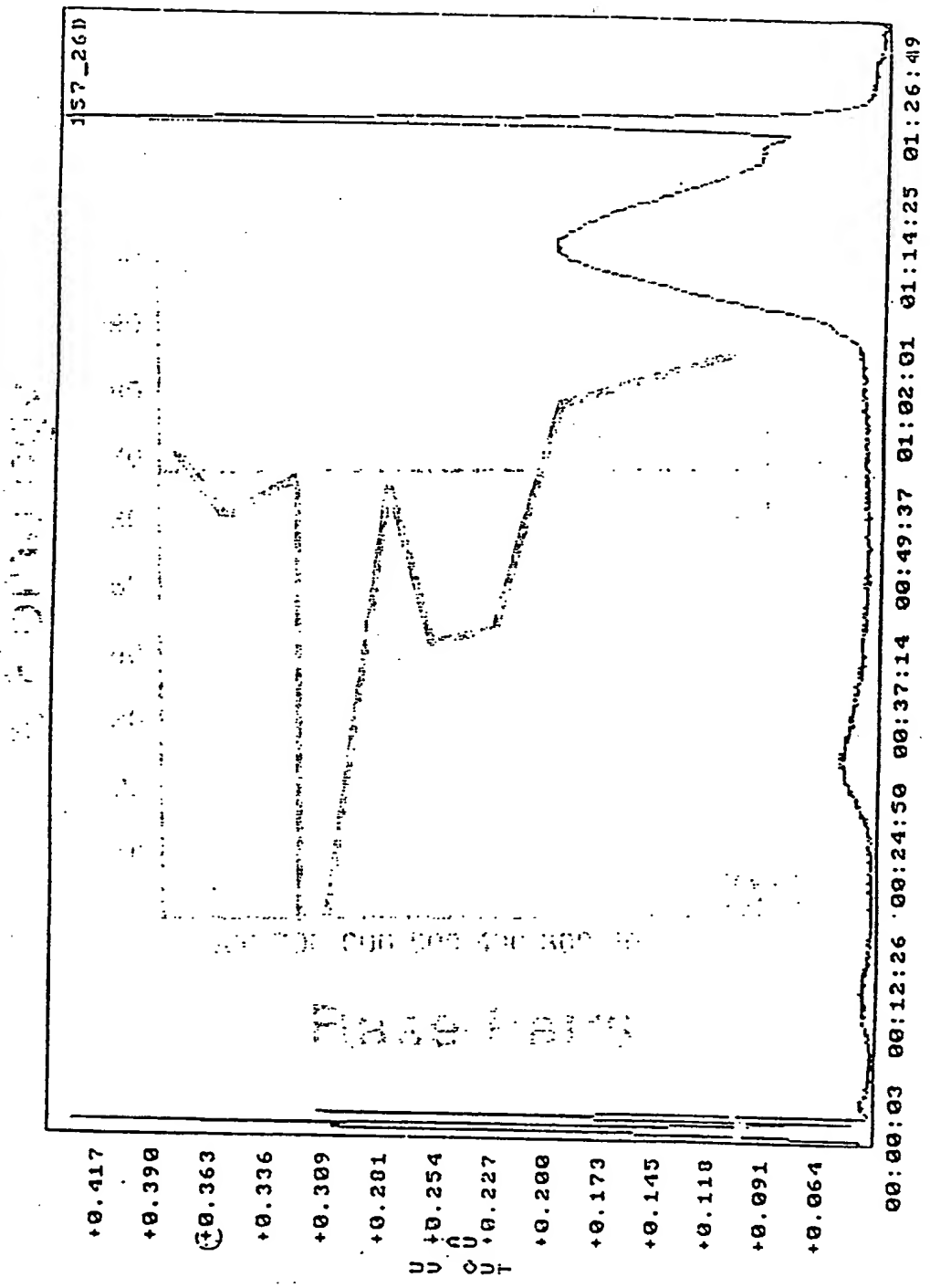

JS7_261
+0.417
+0.390
+0.363
+0.336
+0.309
+0.281
+0.254
+0.227
+0.200
+0.173
+0.145
+0.118
+0.091
+0.064
00:00:03 00:12:26 00:24:50 00:37:14 00:49:37 01:02:01 01:14:25 01:26:49
TIME - hh:mm:ss

FIG. 6

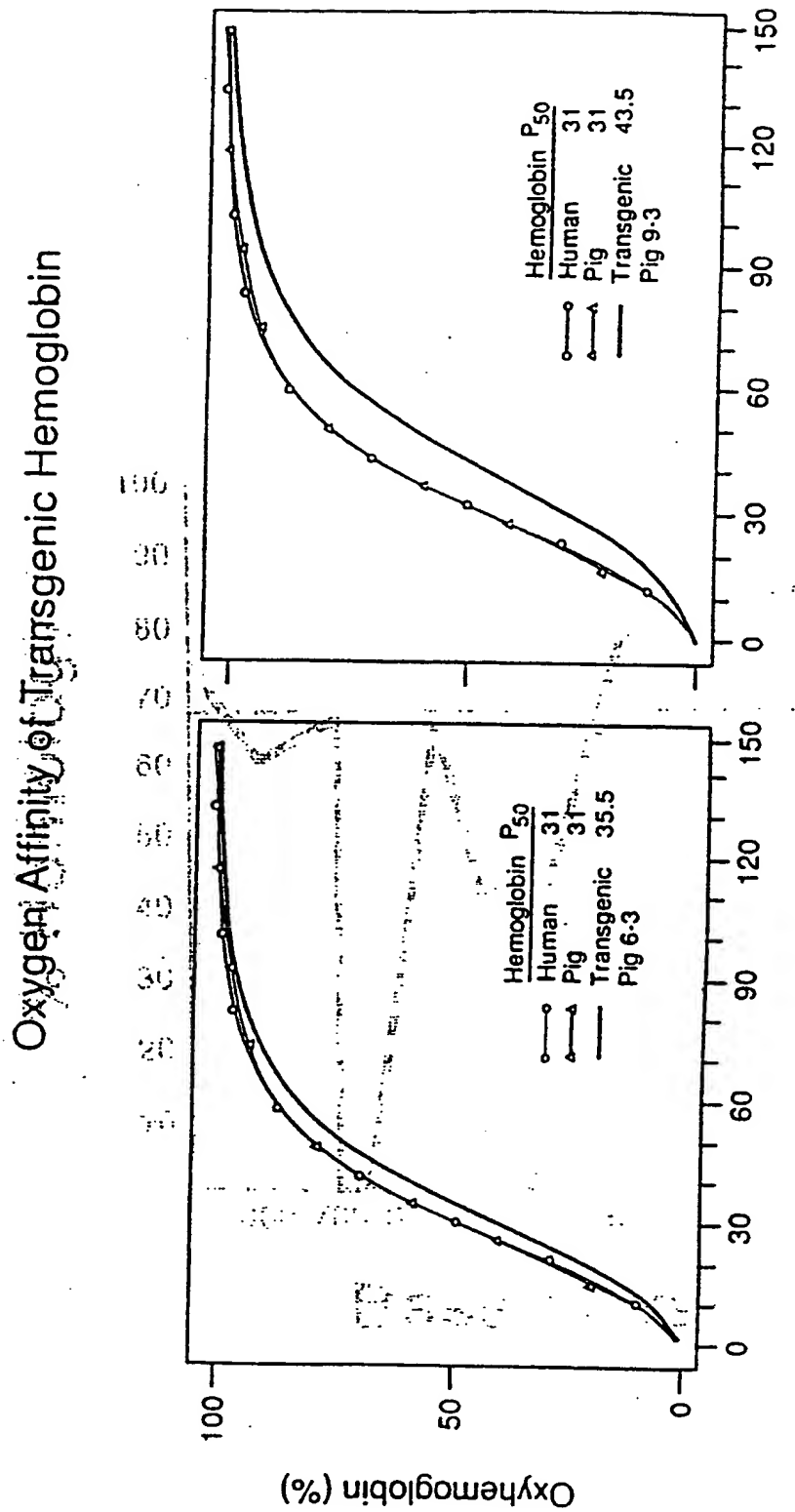

Oxygen Affinity of Transgenic Hemoglobin
Oxyhemoglobin (%)
100
50
0
0
30
60
90
120
150
O_2 (mm of Hg)
Hemoglobin P_{50}
Human 31
Pig 31
Transgenic Pig 6-3 35.5
Hemoglobin P_{50}
Human 31
Pig 31
Transgenic Pig 9-3 43.5

FIG. 7

Adult pig globin promoter

```
        10         20         30         40         50         60
CCCCAGCCCT TTTTCCAGGT CAGCGCAGGG AAAAAACATG TTCTCTGTCC CTGGTTATAC

        70         80         90        100        110        120
TGTTTAGAAA CATCACCTCC CTCGGCGAAA CTAAAACTTG GGGGTTGCAA TTTATTCCTT

       130        140        150        160        170        180
GCTTCTTTGT ATTTCGTACC ACATTGAGAG AGCTCTAGGT TTTCATCCGC AGATTCCCAA

       190        200        210        220        230        240
ACCTTCGCAG AGGAGCTGTT TCACAGGACC GTGATTCAAG TTTACTCTAC TTTTCCATCA

       250        260        270        280        290        300
TTTATTTGGT CATATGTTTA AATGAAGAAA GAAAGGAATG AAGATACCTG AATGAAATGA

       310        320        330        340        350        360
GTATTTGTTT TCTTACCAGC AGGACTGAAT ACAAATGAAG AGAAGAAAAA TACGCACATT

       370        380        390        400        410        420
TAGGACTTGG GCAGAGGTTT TATCCACGCT CTCCTTGTGG TTATTTCCCA TATTCAGAAG

       430        440        450        460        470        480
GCGCGGGTGT GGATTCGTCT GTATGGTCCT AAATTGAACC ACAGTGGTCA AATCCCTCCA

       490        500        510        520        530        540
CTTTCTGCTC CTTGGATTCT TCGTTTGTGT ACTAAGAAAA TGGGGAGGCA GTCTCTAAGA

       550        560        570        580        590        600
GATTGCTACA GTGGGACTCA ACTCTAAAAG TTGTACAGAC TTGCTAAGGA GGATGAAATT

       610        620        630        640        650        660
AGTAGCACTT TGCACTGTGA GGATGGACCT AGAGCTCCCC AGAGAAGGGC TGAAGGTCTG

       670        680        690        700        710        720
AAGTTGGTGC CAGGAACGTC TCGAAGACAG GTATACTGTC AACATTCAAG CCTCACCCTG

       730        740        750        760        770        780
TGGAACCACG CCCTGGCCTG GGCCAATCTG CTCCCAGAAG CAGGGAGGGC AGGAGGCTGG

       790        800        810        820        830        840
GGGGGCATAA AAGGAAGAGC AGAGCCAGCA GCCACCTACA TTTGCTTCTG ACACAACCGT

       850        860        870        880
GTTCACTAGC AACTGCACAA ACAGACAACA TGGTGCATCT GTCTGCTGA
```

Figure 8

```
1       CCCCAGCCCTTTTTCCAGGTCAGCGCAGGGAAAAAACATGTTCTCTGTCCCTGGTTATAC
1287    CCCCAGACACTCTTGCAGATTAGTCCAGGCAGAAA CA GTTAGATGTCCCCAGTTAACC
           *  ** *   *    * *  *  **          *  ***          **   **

61      TG T TTAGAAACATCACCTC CCTCGGCGAAACTAAAACTTGGGGGTTGCAATTTATTC
1345    TCCTATTTGACACCACTGATTACCCCATTGATAGTCACACTTTGGG TTGTAAGTGACTT
        ** *  *   *   ** *** **  * ***  *  *  *  *     *    *  *  * *

118     CTTGCTTCTTTGTATTTCGTACCACATTGAGAGAGCTCTAGGTTTTCATCCGCAGATTCC
1404    TTTATTTATTTGTATTTTTGACTGCATTAAGAGGTCTCTAGTTTTTTATCTCTTGTTTCC
        *   **  *         ***  **    *    *     *      *   **** *

178     CAAACCTTCGCAGAGGAGCTGTTTCACAG G ACCGTGATTCAAGTTTACTCTACTTTTC
1464    CAAAACCTAATA AGTAACTAATGCACAGAGCACATTGATTTGTATTTATTCTATTTTTA
          * * *** *  *  *   ** *        * *  **      ***     *    *   *

236     CATCATTTATTTGGTCATATGTTTAAATGAAGAAA  270
1523    GACATAATTTATTAGCATGCATGAGCAAATTAAGAAA  1559
        **           * *****    ***       *

Matches = 176       Length = 277       Matches/length = 63.5 percent

302     TATTTGTTTTCTTACCAGCAGGACTGAATACAAATGAAGAGAAGAAAAA TACGCAC  A
1629    TTTTTCTTTTCTTACCAGAAGGTTTTAATCCAAATAAGGAGAAGATATGCTTAGAACTGA
         *  *            *   ** *   *                *  ***  **  *   **

359     TTTAGGACTTGGGCAGAGGTTTTATCCACGCTCTCCTTGTGGTTATTTCCCATATTCAGA
1689    GGTAG AGTTTT CATCCATTCTGTCCTGTAAGTATTT TGCATATTCTGGAGACGCAGG
        **    *   ***  ****  * *  ******  **  *   **    **** * **    *

419     AGGCGCGGG TGTGGAT TCGT     CTGTATGGTCCTAAATTGAAC CACAGTGGTCAA
1746    AAGAGATCCATCTACATATCCCAAAGCTGAATTATGGTAGAGAAAGCTCTTCCACTTTTA
         * * ***** *  **    ******   *   ** **   *  *** * * ******* **

472     ATCCCTCCACTTTCTGCTCCTTGGATTCTTCGTTTGTGTACTAAGAAAATGGGGAGGCAG
1606    GTGCATCAA TTTCTTATTTGTGTAATAAGAAAATTGGGAAAACGATCTTCAATATGCTT
        * *  * * *   * ***   *  * ******* **  *  ** *   *** **** *  **

532     TCTCTAA GAGATTGCTAC AGTGGG ACTCA ACTCTAAAAGTTGTACAGACTTGCTAA
1865    ACCAAGCTGTGATTCCAAATATTACGTAAATACACTTGCAAAGGAGGATCTTTTTAGTA
        * ****** *      * * ** * ** * ***  *   ***   ** * *****  ** *

588     GGAGGATGAAATTAGTAGCACTTTGCACTGTGAGG ATGG ACCTAGAGCTCCCCAGAGA
1924    GCAATTTGTACTGA TGGTATGGGGCCAAGAGATATATCTTAGAGGGAGGGCTGAGGGTT
        * ***  * * * * * * * ****  *** *  ***  *** ****   ** **** ***

646     AGGGCTGAAGGTCTGAAGTTGGTGCCAGGAACGTCTCGAAGACAGGTATA CTGTCAACA
1983    TGAAGTCCAACTCCTAAGCCAGTGCCAGAAGAG C CAAGGACAGGTACGGCTGTCATCA
        * ***.** **  **    ***        * ** * * * * *      ***        *

705     TTCAAGCCTCACCCTGTGGAACCACGCCCTGGCCTGGGCCAATCTGCTCCCAGAAGCAGG
2041    CTTAGACCTCACCCTGTGGAGCCACACCCTAGGGTTGGCCAATCTACTCCCAGGAGCAGG
        * * **               *      *   * ** *       *         *

765     GAGGGCAGGAGGCTGGGG GGGCATAAAAGGAAGAGCAGAGCCAGCAGCCACCTACATTT
2101    GAGGGCAGGAGCCAGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTT
                   * *   **            **  *            * ***** *

824     GCTTCTGACACAACCGTGTTCACTAGCAACTGCACAAACAGACAACATGGTGCATCTGTC
2161    GCTTCTGACACAACTGTGTTCACTAGCAAC  CTCAAACAGACACCATGGTGCACCTGAC
                      *                **  *          *          *  *

884     TGCTGA     889
2219    TCCTGA     2224
         *
```

Figure 9.

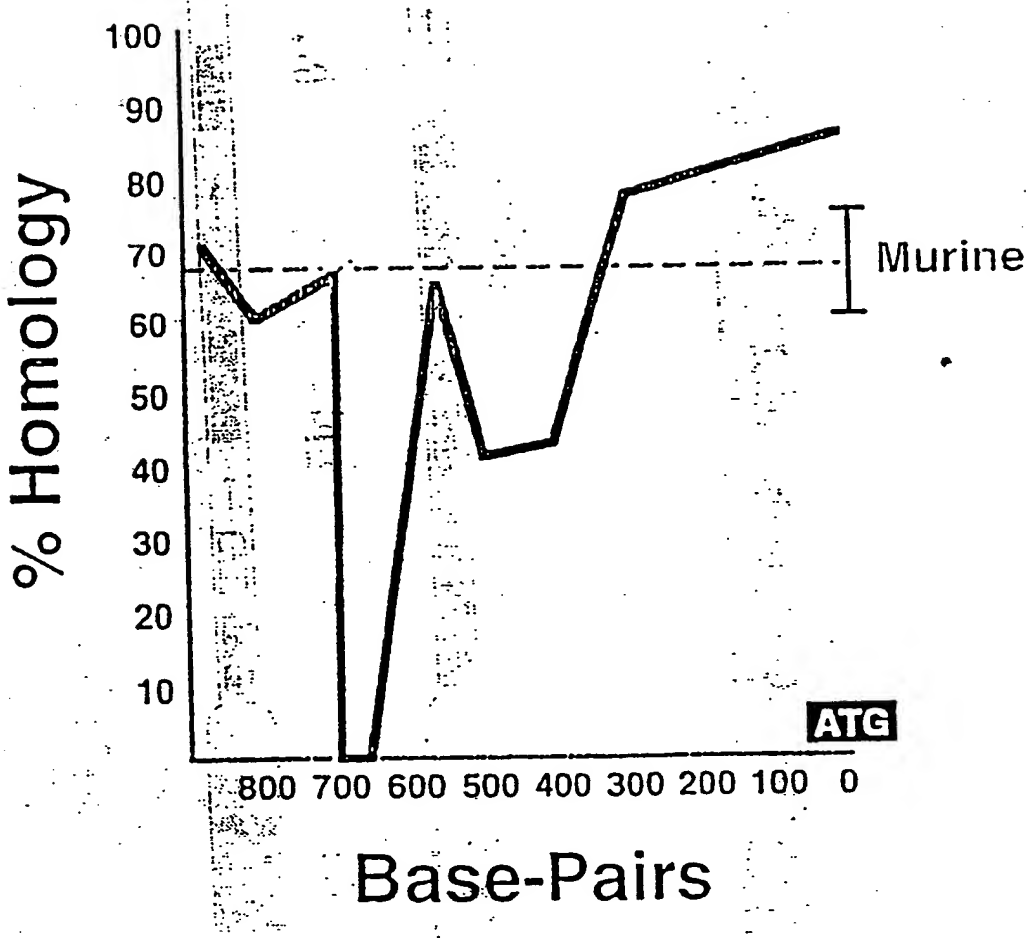

100
90
80
70
60
50
40
30
20
10
% Homology
Murine
ATG
800 700 600 500 400 300 200 100 0
Base-Pairs

Figure 10.

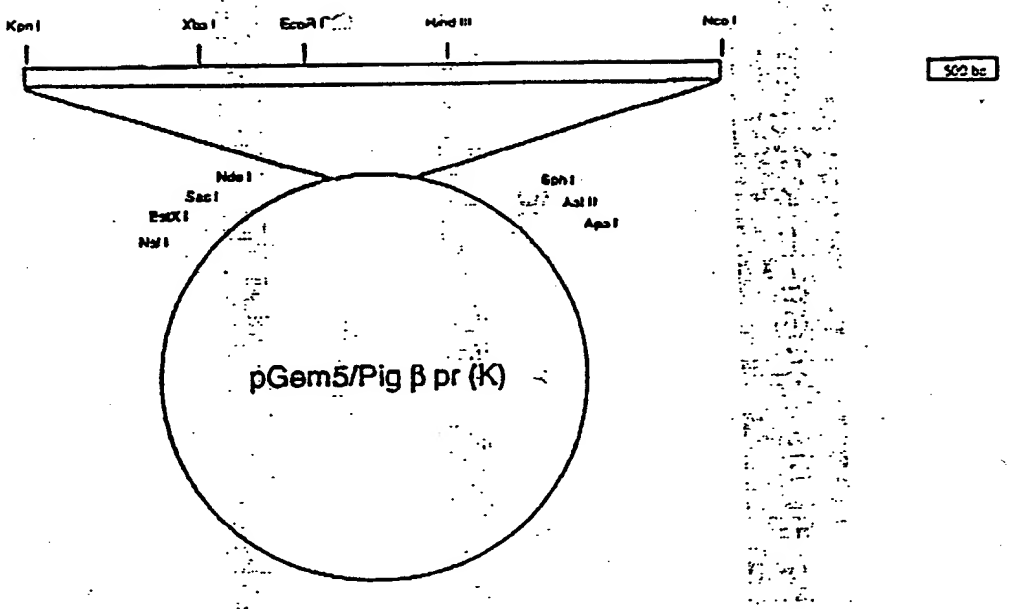
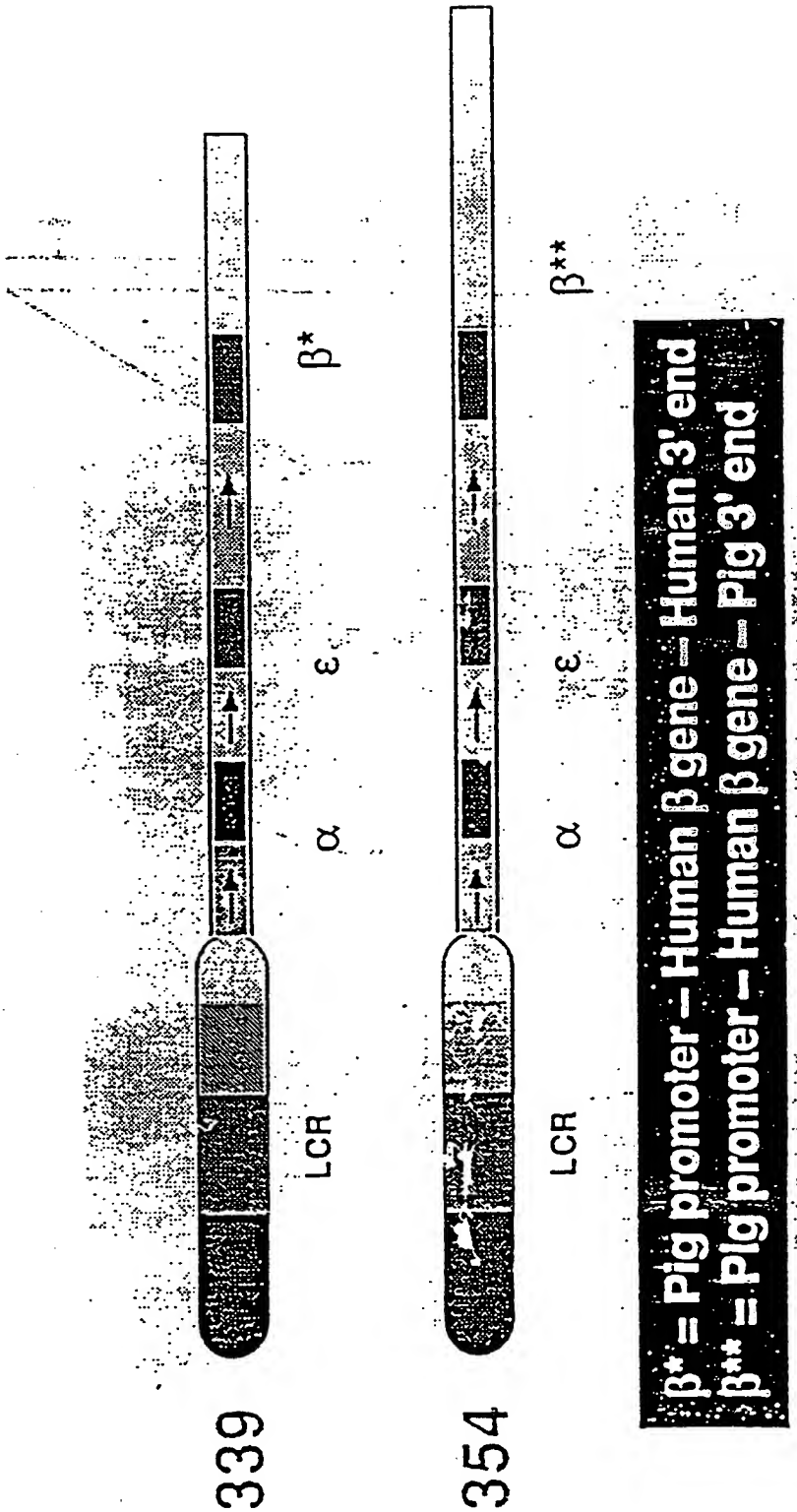

Kpn I
Xba I
EcoR I
Hind III
Nco I
500 bc
Nde I
Sac I
EstX I
Nsi I
Sph I
Ase II
Apa I
pGem5/Pig β pr (K)

Figure 11.

Figure 12


LCR
α
ε
β*
β**
339
354
β* = Pig promoter – Human β gene – Human 3' end
β** = Pig promoter – Human β gene – Pig 3' end

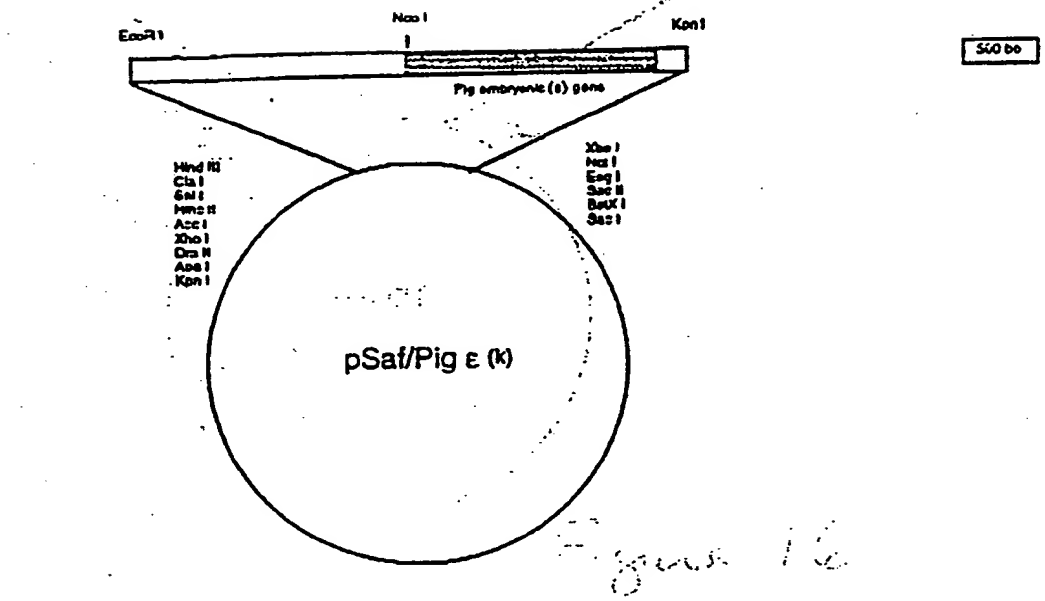
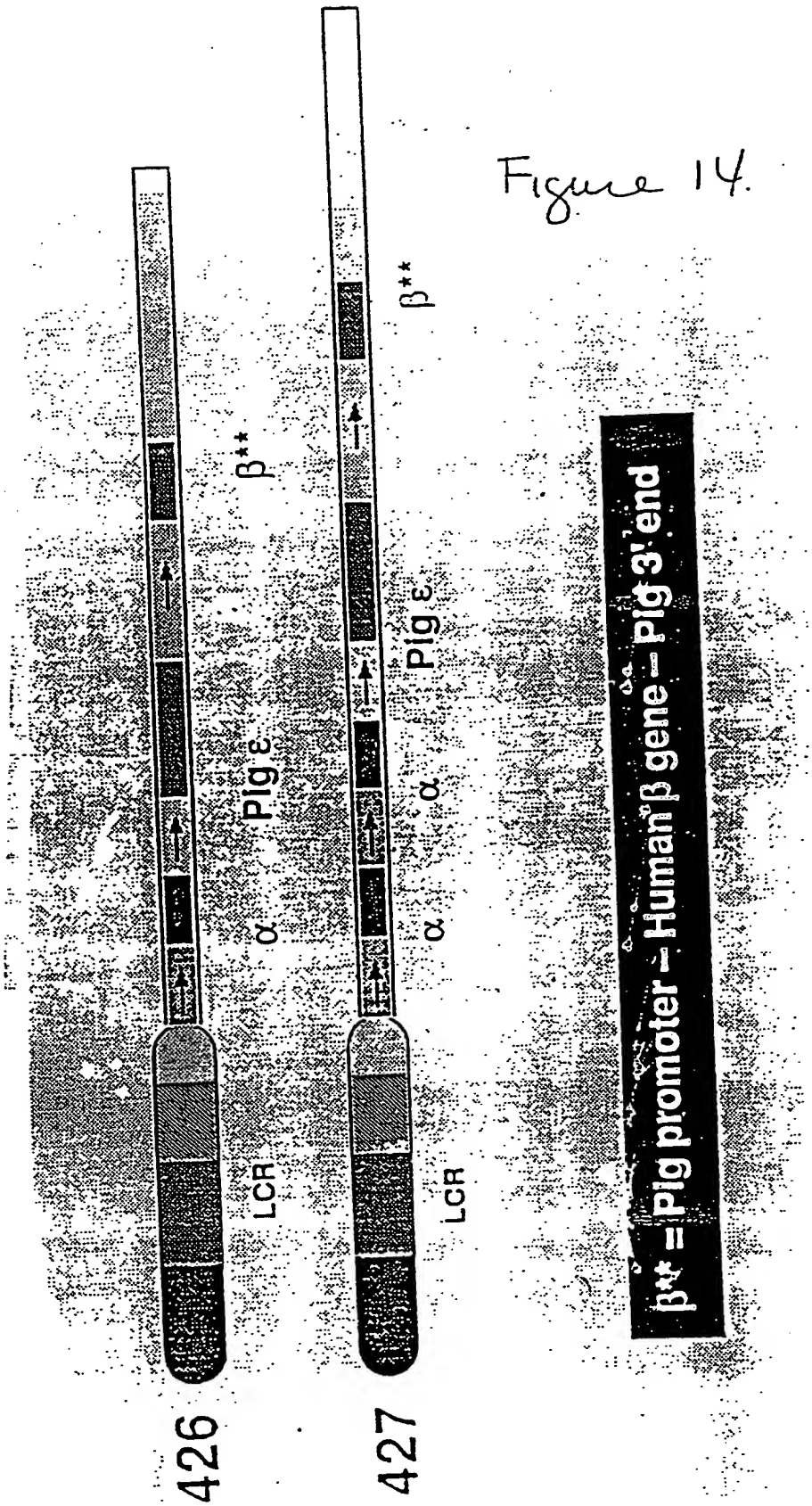


Figure 13.

Figure 14.


β** = Pig promoter – Human β gene – Pig 3' end
426
427
LCR
LCR
α
α
Pig ε
Pig ε
β**
β**

High Level Expression of Hemoglobin (Transgenic Pig)

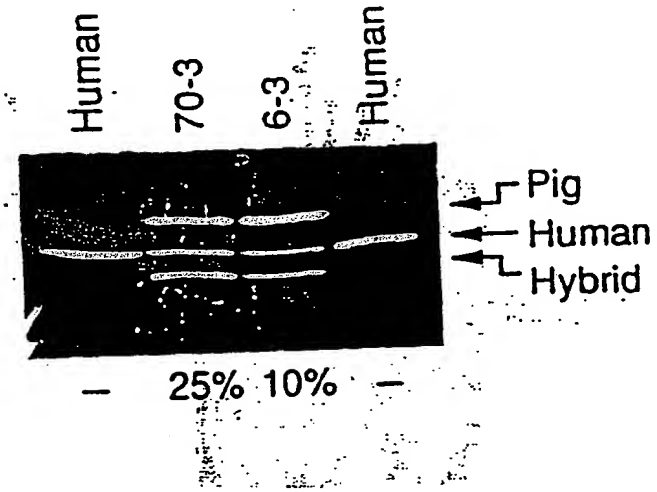


Figure 15

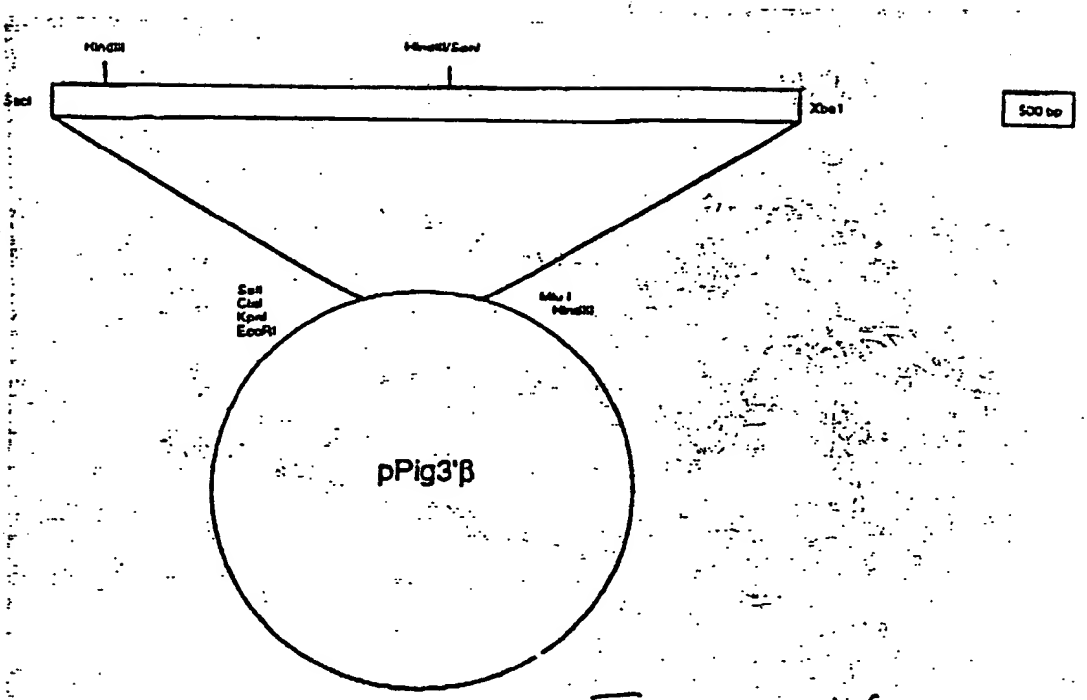

HindIII
HindIII/EcoRI
SacI
XbaI
500 bp
SalI
ClaI
KpnI
EcoRI
MluI
NheI/XbaI
pPig3'β

Figure 16.

FIGURE 17

Transgenic pigs obtained from construct 339

Animal (Sex)	% Authentic Human Hb Expression	Copy #
70-3 (F)	23	3
80-4 (F)	18	3-4
81-3 (F)	5	n.d.

Hb: Hemoglobin

n.d: not determined

FIGURE 18

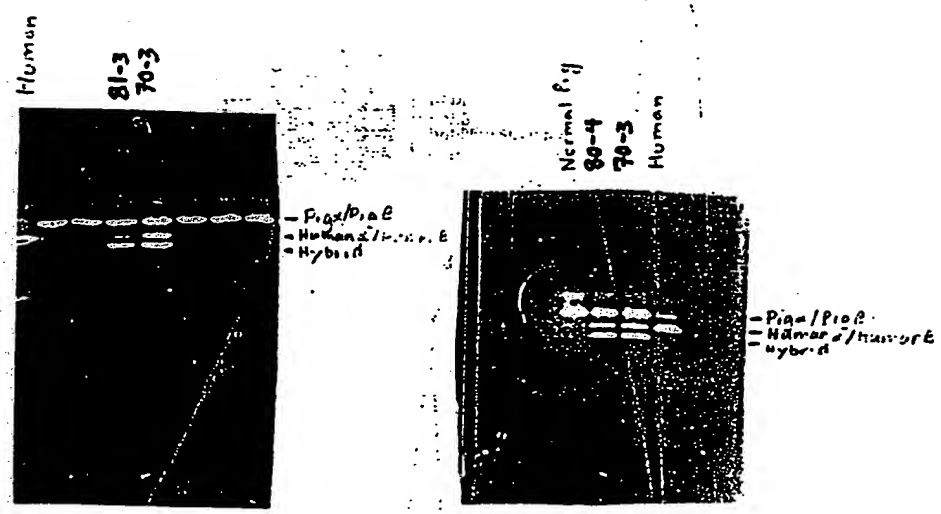


FIGURE 19

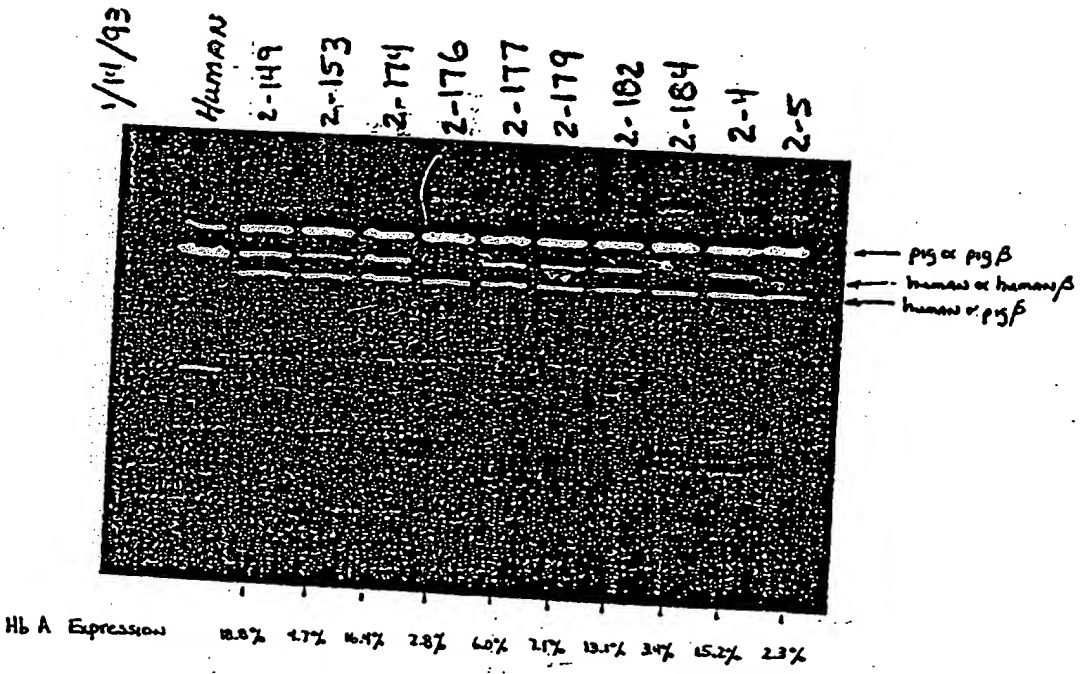

1/11/93
Human
2-149
2-153
2-174
2-176
2-177
2-179
2-182
2-184
2-4
2-5
pig α pig β
human α human β
human α pig β
Hb A Expression
18.8%
4.7%
16.4%
2.8%
6.0%
2.1%
13.1%
3.4%
15.2%
2.3%

FIGURE 20

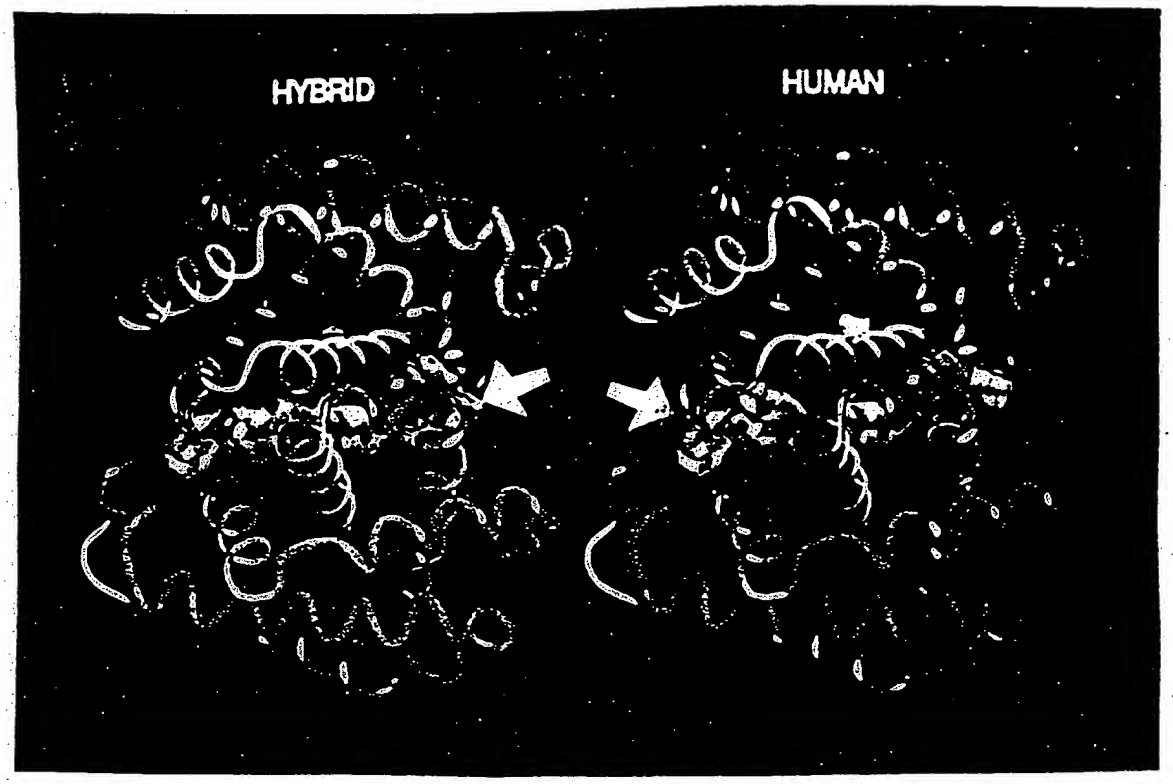


FIGURE 21

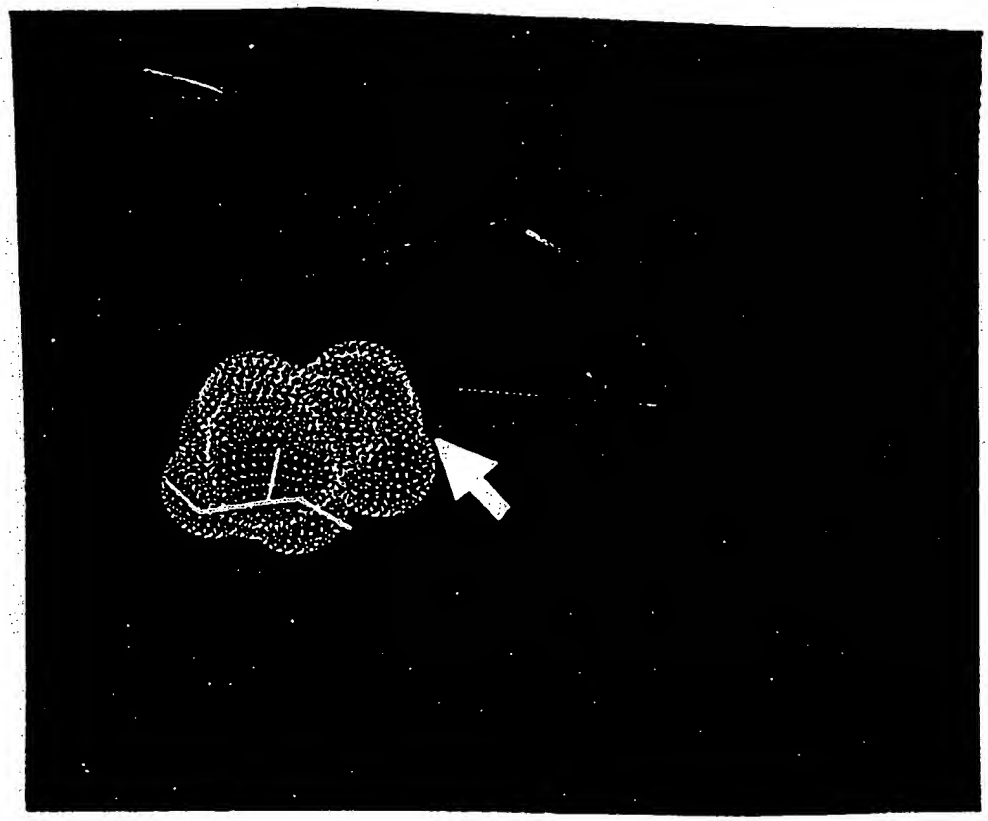


FIGURE 22

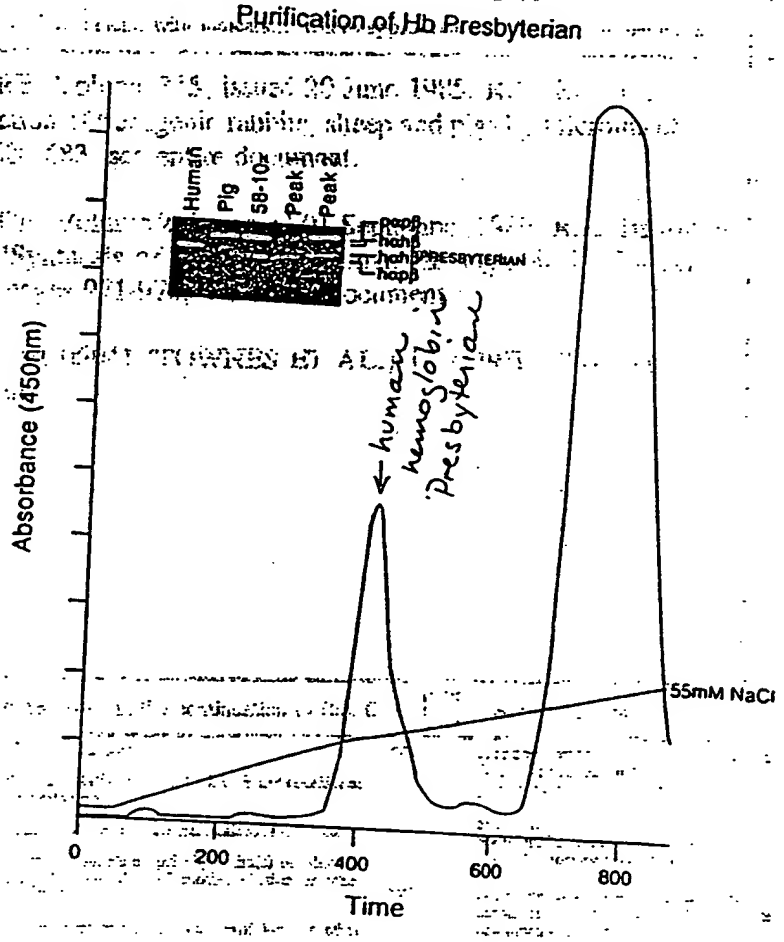

Purification of Hb Presbyterian
Human
Pig
58-10
Peak
Peak
PRESBYTERIAN
Absorbance (450nm)
← human hemoglobin Presbyterian
55mM NaCl
0
200
400
600
800
Time

FIGURE 23

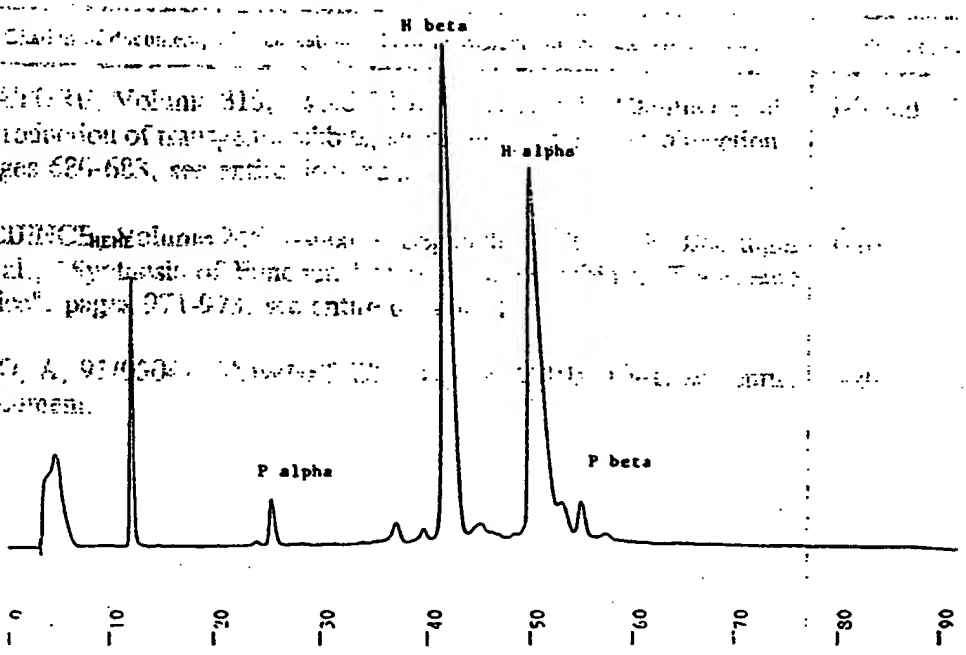
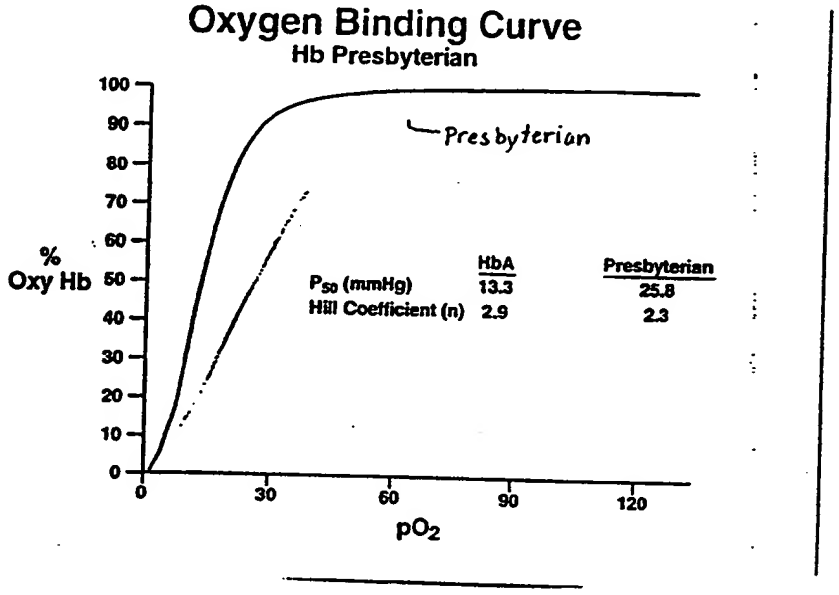
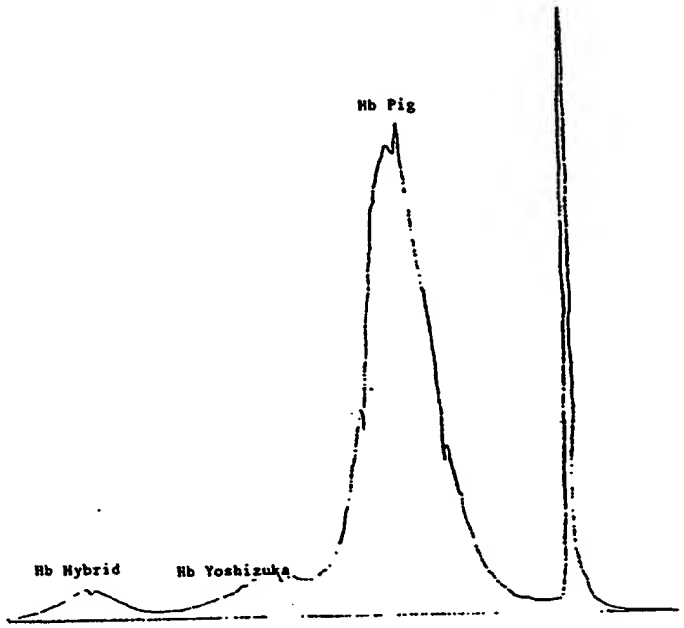


FIGURE 24



C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT 52/52

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE ... ACADEMY OF SCIENCE ... N.J. Pandolfo ... FIGURE 25 ...	11



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/05629

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5): A01K 67/00, 67/027; C12N 15/90; C12P 21/06
US CL: 435/69.1, 69.6; 536/23.1, 23.5, 24.1, 24.2; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.6; 536/23.1, 23.5, 24.1, 24.2; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS, APS, CA
search terms: transgen?; pig#; porcine; hemoglobin; globin; epsilon; purif?; resin#; ion (w) exchange; Q; human; cdna; genom?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 315, issued 20 June 1985, R.E. Hammer et al., "Production of transgenic rabbits, sheep and pigs by microinjection", pages 680-683, see entire document.	1-3 and 13-20
Y	SCIENCE, Volume 245, issued 01 September 1989, R.R. Behringer et al., "Synthesis of Functional Human Hemoglobin in Transgenic Mice", pages 971-973, see entire document.	1-20
Y	WO, A, 91/05041 (TOWNES ET AL.) 18 APRIL 1981, see entire document.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 September 1993	20 SEP 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, Volum 86, issued September 1989, T. Enver et al., "The human ß-globin locus activation region alters the developmental fate of a human fetal globin gene in transgenic mice", pages 7033-7037, see entire document.	1-20
Y	JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, Volume 14, issued 1987, C. Gelfi et al., "Purification of human hemoglobin valence intermediates by preparative immobilized pH gradients", pages 129-147, see entire article.	18 and 20
Y	JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, Volume 17, issued 1988, S.M. Christensen et al., "Preparation of human hemoglobin Ao for possible use as a blood substitute", pages 143-154, see entire article.	1-20
Y	JOURNAL OF CHROMOTOGRAPHY, volume 487, issued 1989, F. Kutlar et al., "QUANTITATION OF HEMOGLOBIN BART'S, H, PORTLAND-I, PORTLAND-II AND CONSTANT STRING BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMOTOGRAPHY", pages 265-274, see entire article.	18 and 20
Y	JOURNAL OF CHROMOTOGRAPHY, volume 427, issued 1988, C.T.A. Evelo et al., "Separation of human haemoglobin alkylated at ß93 cysteine from its native form by fast protein liquid chromotography", pages 335-340, see entire article.	18 and 20
Y	JOURNAL OF CHROMOTOGRAPHY, volume 359, issued 1986, D.J. Burke et al., "RAPID CATION-EXCHANGE CHROMOTOGRAPHY OF HEMOGLOBINS AND OTHER PROTEINS", pages 533-540, see entire article.	18 and 20
Y	E. ANTONINI et al. "METHODS IN ENZYMOLOGY, VOLUME 76, HEMOGLOBINS", published 1981 by ACADEMIC PRESS (N.Y.), see pages 97-125, see entire excerpt.	18 and 20
Y	CELL, volume 38, issued August 1984, S. Wright et al., "DNA Sequences Required for Regulated Expression of ß-Globin Genes in Murine Erythroleukemia Cells", pages 265-273, see entire article.	10-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05629

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, volume 76, number 11, issued November 1979, N.J. Proudfoot et al., "Molecular cloning of human epsilon-globin gene", pages 5433-5439, see entire article.	11.

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05629

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-3 and 13-20, drawn to transgenic pigs and methods of making hemoglobin, classified in Class 800, subclass 2 and Class 435, subclass 69.6.

II. Claim 10, drawn to a ß-globin promoter, classified in Class 536, subclass 24.1.

III. Claim 11, drawn to a pig epsilon gene, classified in Class 536, subclass 23.5.

IV. Claim 12, drawn to the 3'-non-coding region of the pig adult ß-globin gene, classified in Class 24.1.

The inventions are distinct, one from the other for the following reasons:

The invention of group I is distinct from the inventions of Groups II-IV because they are drawn towards materially different compositions. For example, the compositions of group I comprise transgenic pigs whereas the compositions of the other three groups are drawn to nucleic acids. Further, the transgenic compositions are characterized in that they express human hemoglobin genes while the nucleic acid compositions of groups II-IV are derived from porcine genes.

The inventions of Groups II-IV are distinct one from the other because they are drawn to materially different elements of porcine nucleic acid. For example, the nucleic acid of group I comprises the promoter region for the porcine ß-globin gene, whereas the nucleic acid of Group II comprises the structural gene for the porcine epsilon gene which is chemically unrelated to the ß-globin locus. The invention of Group IV is directed towards a non-coding region of the porcine ß-globin gene which does not mediate any physical process such as transcription and is therefore distinct from the promoter region of Group II,

In addition, the compositions of Groups II-IV may be used for materially different purposes other than the generation of transgenic animals, such as the production of recombinant proteins *in vitro*. Therefore, the four inventions listed above lack any special technical feature within the meaning of PCT Rule 13.2, linking them so as to constitute a unified invention.

Appendix D

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11153–11157, November 1994
Applied Biological Sciences

Expression of a functi nal human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection

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Communicated by George E. Seidel, Jr., June 23, 1994

ABSTRACT **The serious shortage of human organs available for transplantation has engendered a heightened interest in the use of animal organs (xenografts) for transplantation. However, the major barrier to successful discordant xenogeneic organ transplantation is the phenomenon of hyperacute rejection. Hyperacute rejection results from the deposition of high-titer preformed antibodies that activate serum complement on the luminal surface of the vascular endothelium, leading to vessel occlusion and graft failure within minutes to hours. Although endogenous membrane-associated complement inhibitors normally protect endothelial cells from autologous complement, they are species restricted and thus confer limited resistance to activated xenogeneic complement. To address the pathogenesis of hyperacute rejection in xenotransplantation, transgenic mice and a transgenic pig were engineered to express the human terminal complement inhibitor hCD59. High-level cell surface expression of hCD59 was achieved in a variety of murine and porcine cell types, most importantly on both large vessel and capillary endothelium. hCD59-expressing porcine cells were significantly resistant to challenge with high-titer anti-porcine antibody and human complement. These experiments demonstrate a strategy for developing a pig-to-primate xenogeneic transplantation model to test whether the expression of a human complement inhibitor in transgenic pigs could render xenogeneic organs resistant to hyperacute rejection.**

The lack of effective therapies aimed at eliminating antibody- and complement-mediated hyperacute rejection presents a major barrier to the successful transplantation of discordant animal organs into human recipients (1–6) and has precluded the development of animal models aimed at evaluating the *in vivo* cellular immune response to discordant xenografts. Old World primates, including humans, have high levels of preexisting circulating natural antibodies that predominantly recognize carbohydrate determinants expressed on the surface of xenogeneic cells from discordant species (2–6). Recent evidence indicates that most of these antibodies react with the carbohydrate epitope, Gal(α1-3)Gal (7), an epitope absent from Old World primates because of a lack of the functional α-1,3-galactosyltransferase enzyme (8). Therefore, after transplantation of a vascularized xenogeneic donor organ into a primate recipient, the massive inflammatory response that ensues from natural antibody activation of the classical complement cascade leads to activation and destruction of the vascular endothelial cells and ultimately of the donor organ within minutes to hours after revascularization (2–6). Endogenously expressed membrane-associated complement regulatory proteins normally protect endothelial cells from autologous complement. However, the activity of these complement inhibitors is species restricted. This property makes them relatively ineffective at inhibiting xenogeneic serum complement (9, 10). The demonstration that a human complement inhibitor could protect a xenogeneic cell from human complement-mediated lysis showed that it was possible to inhibit human anti-porcine hyperacute rejection in *in vitro* models (11).

The strategy used to address the pathogenesis of hyperacute rejection in the porcine-to-primate xenotransplantation model was to produce transgenic swine expressing high levels of the human terminal complement inhibitor hCD59. hCD59 is an 18- to 20-kDa glycosyl-phosphatidylinositol-anchored cell surface glycoprotein that is expressed in a variety of tissues of both hematopoietic and nonhematopoietic lineage and functions to inhibit formation of the membrane attack complex by binding to membrane C5b-8 and C5b-9 (9, 10). Stable expression of hCD59 on xenogeneic cells *in vitro* protected the cells from human complement-mediated cell lysis (12–14) and the level of protection was directly proportional to the number of molecules of hCD59 expressed on the surface of the xenogeneic cell (14). Importantly, hCD59-expressing porcine aortic endothelial cells were resistant not only to cell lysis but also to complement-mediated formation of a procoagulant surface when challenged with either human or baboon serum (15). Taken together, these results indicated that high-level expression of hCD59 could provide porcine tissue with significant protection from human serum complement in a xenotransplantation setting. Therefore, hCD59 was chosen as a candidate molecule for production of transgenic swine resistant to human complement. In this report, we demonstrate the successful production of a transgenic pig expressing high levels of hCD59 that protect the pig cells from human complement-mediated cell lysis.

MATERIALS AND METHODS

***H2K^b*-hCD59 DNA Construct, Purification, and Microinjection.** A hCD59 cDNA was directionally cloned into exon 1 of the murine *H2K^b*-gene 12 nucleotides downstream of the transcriptional start site. Briefly, the hCD59 cDNA fragment was excised from a hCD59-pcDNAI-Amp (pcDNAI-Amp; Invitrogen) expression plasmid by digestion with *Hin*dIII, followed by enzymatically filling in the 5′ 4-nucleotide overhang with T4 DNA polymerase and dNTPs. Subsequently,

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Abbreviations: MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; mAb, monoclonal antibody; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; hrTNF-α, human recombinant tumor necrosis factor α; IFN, interferon.

†To whom reprint requests should be addressed.

the DNA was digested with *Not* I at the 3′ end of the multiple cloning site of the vector to yield a 452-bp cDNA fragment. The 9.0-kbp *Eco*RI $H2K^b$ genomic restricti n fragment (16) cloned into pGEM7Z (Promega) was digested with *Nru* I and *Not* I, resulting in the removal f 51 nucleotides from the $H2K^b$ gene including the ATG start codon. The hCD59 cDNA was then directionally ligated into the $H2K^b$ gene in the pGEM7Z vect r.

Purification of the $H2K^b$–hCD59 DNA f r embryo injection was accomplished by digesting the plasmid with *Xho* I to remove the vector sequences followed by agarose gel electrophoresis, electroelution, and Elutip purification (Schleicher & Schull). Transgenic mice were produced by pronuclear microinjection of murine ova as described (17). Ten of 60 offspring were identified as transgenic founder animals by DNA slot blot hybridization (18) (data not shown). Transgenic swine were generated by porcine embryo injection (19). A total of 18 piglets were analyzed by DNA slot blot analysis of genomic DNA (18). One founder animal, $H2K^b$–hCD59 153-2, contained 10–20 copies of the $H2K^b$–hCD59 DNA. Two additional founder animals, $H2K^b$–hCD59 152-1 and $H2K^b$–hCD59 152-2, contained ≈1 copy of the $H2K^b$–hCD59 DNA and exhibited no expression or very low and inconsistent levels of expression in peripheral blood mononuclear cells (PBMCs) (data not shown). These animals were not analyzed further.

Cell Culture, Immunofluorescence, and Immunohistochemistry. PBMCs from transgenic and negative littermate control pigs were purified from whole blood by Ficoll gradient centrifugation (ref. 20, pp. 7.1.1–7.1.2). Adherent monocytic mononuclear cells were cultured in Dulbecco's modified Eagle's medium/15% fetal bovine serum. PBMCs from transgenic mice and negative littermate control animals were purified from whole blood by ACK lysis (Biofluids, Rockville, MD). Indirect immunofluorescence of porcine PBMCs was performed with the anti-hCD59 mouse monoclonal antibody (mAb) MEM-43 (Biodesign International, Kennebunkport, ME) and with the anti-swine leukocyte antigen (SLA) class I mAb PT85A (VMRD, Pullman, WA). Indirect immunofluorescence of murine PBMCs was performed with polyclonal antisera specific for hCD59 (P. Sims, Blood Research Institute, Milwaukee). Goat anti-rabbit IgG (polyclonal sera; Zymed) or goat anti-mouse IgG (monoclonal sera; Zymed) fluorescein isothiocyanate (FITC)-conjugated antisera were used to detect specific antibody binding to the cell surface. Cell surface expression was then measured by flow cytometry on a Becton Dickinson FACSort.

The cytokine inducibility of $H2K^b$–hCD59 and the endogenous porcine SLA class I molecule was tested on adherent peripheral blood monocytes. Briefly, porcine cytokine-conditioned medium supernatants were produced from control pig PBMCs. PBMCs harvested from a control pig were stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 48 h. PHA-conditioned media were collected and treated with 10 mM methyl α-mannoside and filter sterilized. Human recombinant tumor necrosis factor α (hrTNF-α; Collaborative Biomedical Products, Bedford, MA) was used at 500 units/ml. Adherent peripheral blood monocytes were then treated with medium alone, 50% PHA-conditioned medium (diluted 1:1 with complete medium), 50% PHA-conditioned medium/hrTNF-α, or hrTNF-α for 24 h. Cytokine-induced expression of hCD59 and SLA class I was detected by immunofluorescence and fluorescence-activated cell sorter analysis as described above.

Immunohistochemistry was performed on fresh frozen sections embedded in Tissue-Tek OCT compound (Miles). Tissue sections (5–10 μm) were processed as described (ref. 20, pp. 5.8.1–5.8.2). Sections that were double stained were processed simultaneously with the mouse anti-hCD59 mAb, MEM-43 (20 μg/ml), and the anti-type IV collagen rabbit polyclonal antiserum (21) (1:50 diluti n). Fluorochrome-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antisera were used to detect specific antibody interactions with the hCD59 antigen (goat anti-mouse rhodamine; AMAC, Westbrook, ME) and type IV collagen antigen (goat anti-rabbit FITC; Zymed).

Complement-Mediated Dye Release Assays. PBMCs or peripheral blood adherent cells were labeled with the intracellular dye Calcein AM (Molecular Probes). The cells were subsequently incubated with anti-porcine blood cell IgG (2 mg/ml) (Intercell Technologies, Hopewell, NJ) followed by incubation in increasing concentrations of human whole serum (Sigma) at 37°C for 30 min. Dye released from the cells was determined by flow cytometry on a Becton Dickinson FACSort. The C5b-9-specific dye release was calculated as percentage of total, correcting for nonspecific dye release and background fluorescence measured on identically matched controls without the addition of serum. Antibody blocking experiments were performed by the complement-mediated dye release assay as described above with the following exceptions. The cells were incubated in 20% C8-deficient serum (C8d; Quidel, San Diego) at 37°C for 30 min after anti-porcine blood cell antibody activation. The cells were then incubated with hCD59 polyclonal antiserum (100 μg/ml) or anti-SLA class I antiserum PT85A (100 μg/ml). Purified human C8 (Quidel) and C9 (Quidel) complement components were then added in increasing concentrations and incubated at 37°C for 30 min. Dye released from the cells was detected by flow cytometry on a Becton Dickinson FACSort as described above.

RESULTS

Transgenic Expression. To achieve expression of the transgene-encoded hCD59 we engineered a murine major histocompatibility complex (MHC) class I gene, $H2K^b$ (16), to control the expression of a hCD59 cDNA, $H2K^b$–hCD59 (Fig. 1). The MHC class I gene is ubiquitously expressed on most somatic cells and, most importantly, is a predominant endothelial cell surface antigen (22, 24). In addition, the MHC class I promoter contains cis-acting regulatory elements that bind cytokine-inducible trans-acting factors, resulting in up-regulation of the class I gene upon stimulation with interferon (IFN)-α/β, IFN-γ, and TNF-α (22–25). A hCD59 cDNA was cloned into exon I of $H2K^b$ and results in a transcript that initiates at the $H2K^b$ transcriptional start site and proceeds through both the cDNA insert and the entire transcriptional unit of the $H2K^b$ gene. Translation initiates at the ATG codon of the inserted cDNA and terminates at the cDNA stop codon. The rest of the $H2K^b$ gene remains untranslated and functions only in RNA processing, providing the cDNA with a genomic structure that contains all the regulatory elements required for $H2K^b$ expression (22–25).

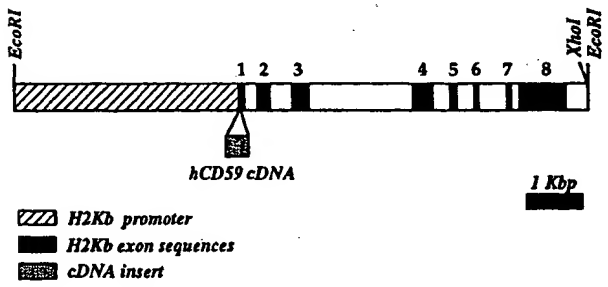

FIG. 1. $H2K^b$ genomic cassette. A linear representation of the hybrid gene construct detailing the exon–intron structure of $H2K^b$ and the insertion of the hCD59 cDNA into exon 1.

The efficacy of the *H2K*^{b}–hCD59 genomic expressi n construct in directing cell surface expression of hCD59 in various tissues was tested in transgenic mice and pigs. Initial analysis demonstrated that the *H2K*^{b}–hCD59 genomic construct directed the expression of hCD59 on the surface of PBMCs in several founder transgenic mice and transgenic pig 153-2 (Fig. 2 *A* and *B*, respectively). Importantly, expression of hCD59 on the surface of the porcine mononuclear cells paralleled that of SLA class I (Fig. 2*B*). The comparable expression of hCD59 to SLA class I indicated that the murine

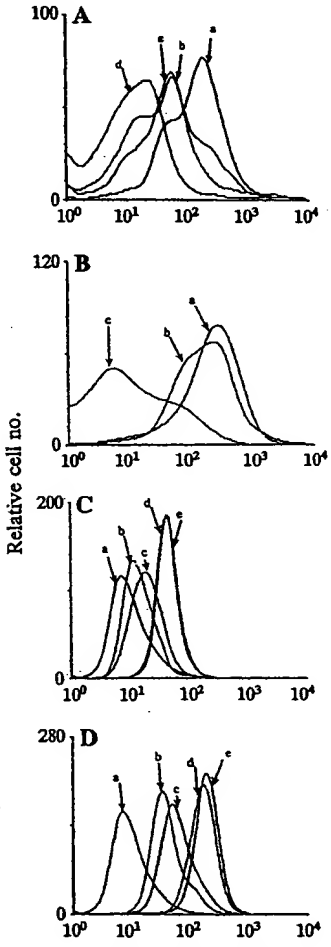

FIG. 2. Cell surface expression of hCD59 in transgenic mice and a transgenic pig. (*A*) Expression of hCD59 on murine PBMCs detected in transgenic mice *H2K*^{b}–CD59-11 (curve a), *H2K*^{b}–CD59-23 (curve b), *H2K*^{b}–CD59-21 (curve c), and a negative littermate control (curve d). (*B*) Cell surface expression of hCD59 and SLA class I detected on porcine PBMCs. Curve a, hCD59 expression in transgenic pig *H2K*^{b}–hCD59 153-2; curve b, SLA class I expression in transgenic pig *H2K*^{b}–hCD59 153-2; curve c, negative littermate control PBMCs incubated with the hCD59 mAb. (*C*) Cytokine-induced cell surface expression of hCD59 on cultured adherant PMBCs from pig *H2K*^{b}–hCD59 153-2; goat-anti-mouse FITC control antisera (curve a); hCD59 expression on uninduced cells (curve b); hrTNF-α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF-α (curve e). (*D*) Cytokine-induced cell surface expression of SLA class I on cultured adherent PBMCs from pig *H2K*^{b}–hCD59 153-2; goat-anti-mouse FITC control antiserum (curve a); uninduced cells (curve b); hrTNF-α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF-α (curve e).

H2K^b–hCD59 chimeric gene was constitutively regulated, similar to the endogenous porcine SLA class I molecules. To establish whether the *H2K*^{b}–hCD59 chimeric gene exhibited cytokine inducibility comparable to the end genous SLA class I gene, we cultured adherent m nocytic PBMCs. Interestingly, after prolonged culture, these m nocytes had downregulated cell surface expression of both SLA class I as well as the hCD59 transgene-encoded protein (compare Fig. 2*B*, curve b, to Fig. 2*D*, curve b for class I and Fig. 2*B*, curve a, to Fig. 2*C*, curve b, for hCD59). Treatment of the transgenic porcine cells with PHA-induced cytokine-conditioned medium, with hrTNF-α, or with a combination of the treatments resulted in an increase in hCD59 expression (Fig. 2*C*) as well as an increase in SLA class I expression (Fig. 2*D*).

We next examined hCD59 expression on the endothelium of vascularized organs. Immunohistochemical analyses were performed on fresh-frozen tissue sections derived from hCD59 transgenic mice and pigs as well as from nontransgenic littermates. Phase-contrast micrographs illustrating the structure of mouse myocardium are shown in Fig. 3 *A* and *D*. Tissue sections from three founder mice were analyzed for hCD59 expression. Mouse hearts were incubated with anti-collagen type IV polyclonal antisera to detect basement membrane structures underlying the endocardium as well as intramyocardial capillary endothelia (21). Fig. 3 *B* and *E*, respectively, confirmed equivalent collagen staining in the negative littermate control and a representative hCD59 transgenic mouse, *H2K*^{b}–hCD59-8. In contrast, staining with a mAb specific for hCD59 revealed intense cell surface expression on endothelial cells in the heart of transgenic mouse *H2K*^{b}–hCD59-8 (Fig. 3*F*) and an absence of hCD59 expression in the negative littermate control (Fig. 3*C*). Fig. 3*F* dramatically highlights the expression of hCD59 on vascular structures and clearly shows high-level expression of hCD59 on the endocardium in the ventricular chamber. Abundant hCD59 was also detected on capillary vessels within the myocardium (Fig. 3*F*). All three founder transgenic mice analyzed revealed hCD59 staining on the endocardium and capillary endothelium. To evaluate vascularized structures in the transgenic pig without having to sacrifice the founder animal, tail sections were prepared and analyzed by immunohistochemistry as described for the mice. Phase-contrast micrographs illustrate the morphological structure of a tail artery from a negative control pig (Fig. 4*A*) and a tail artery

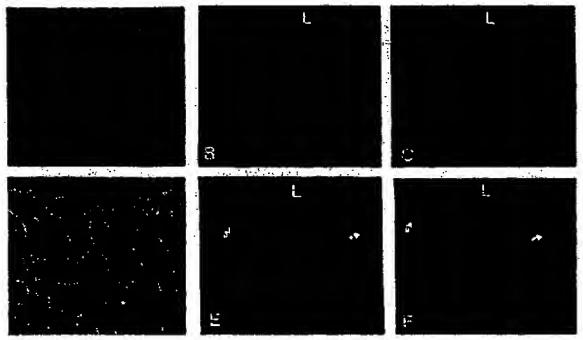

FIG. 3. Double-label immunofluorescence microscopy of hCD59 and type IV collagen on murine heart tissue from a *H2K*^{b}–hCD59 transgenic mouse and a negative littermate control. Phase-contrast micrographs of murine ventricular myocardium (*A* and *D*). L, lumen of the left ventricle lined by endothelial cells. (*B* and *E*) Immunofluorescence micrographs detecting type IV collagen (fluorescein) of the same myocardial sections illustrating basement membrane structures underlying the endocardium. Immunofluorescence micrographs (rhodamine) of the same myocardial sections detecting hCD59 in a negative littermate control (*C*) and *H2K*^{b}–hCD59-8 (*F*). (×400.) (Bar = 25 μm.)

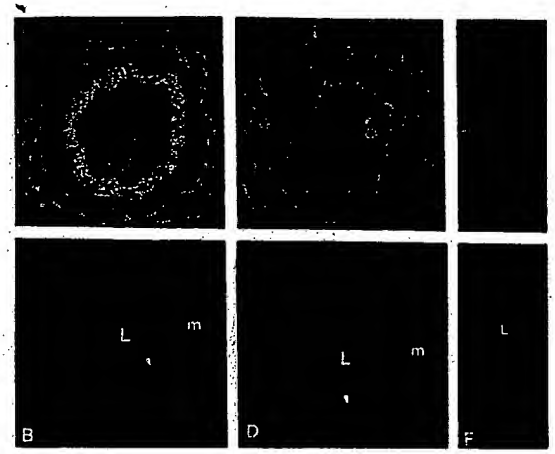

FIG. 4. Immunofluorescence microscopy of hCD59 on swine tail sections from pig *H2K*^{b}–hCD59 153-2 and a negative littermate control. (*A*) Phase-contrast micrograph of a dermal artery from the negative littermate illustrating the lumen (L), the endothelial cell layer (arrow), and the tunica media (m). (*B*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *A*, illustrating the lumen, the endothelial cell layer, and the tunica media. (*C*) Phase-contrast micrograph of a dermal artery from pig *H2K*^{b}–hCD59 153-2, illustrating the lumen, the endothelial cell layer, and the tunica media. (*D*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *C*, illustrating the lumen, the endothelial cell layer, and the medial smooth muscle cells (m). (*E*) Phase-contrast micrograph of a dermal microvessel from pig *H2K*^{b}–hCD59 153-2, illustrating the lumen and the vessel wall. (*F*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *E*, illustrating the lumen, and an abundance of hCD59 expression. (×400.) (Bar = 25 μm.)

and small vessel from the transgenic founder pig 153-2 (Fig. 4 *C* and *E*, respectively). High-level hCD59 expression was observed on a variety of tissue and cell types, including fibroblasts, epithelial cells, vascular endothelial cells, and smooth muscle cells within the tail section of the transgenic pig (Fig. 4 *D* and *F*) but not in the negative littermate (Fig. 4*B*). Not all tissue in the transgenic pig tail section revealed hCD59 staining; however, tissues such as striated muscle are known to express very low levels of the class I antigen and therefore would not be expected to express the class I-regulated hCD59 transgene (24). These analyses confirmed that the *H2K*^{b}–hCD59 genomic construct directed expression of hCD59 to a variety of cells and tissues in transgenic pig 153-2 and, most importantly, to the surface of vascular endothelial cells.

Complement Resistance. To determine whether the high levels of transgene expression observed on the transgenic pig cells conferred significant protection from human complement-mediated attack, functional analyses were performed n hCD59-expressing porcine PBMCs collected from transgenic pig 153-2 and a nontransgenic littermate control. The data clearly demonstrated that hCD59-expressing porcine cells, but not cells from a nontransgenic littermate, significantly resisted human complement-mediated lysis (Fig. 5*A*). The percentage dye released from hCD59 protected cell was ≈5-fold less when compared with the amount of dye released from negative littermate control cells. To confirm that the protection observed in PBMCs was due specifically to hCD59 expression, antibody blocking experiments were performed. As shown in Fig. 5*B*, the anti-hCD59 polyclonal antisera blocked the hCD59-mediated protection, resulting in an increased susceptibility of the porcine cells to human complement-mediated cell lysis. In contrast, the control antibody had no effect.

To evaluate whether the degree of protection of porcine cells from human complement attack was a function of the

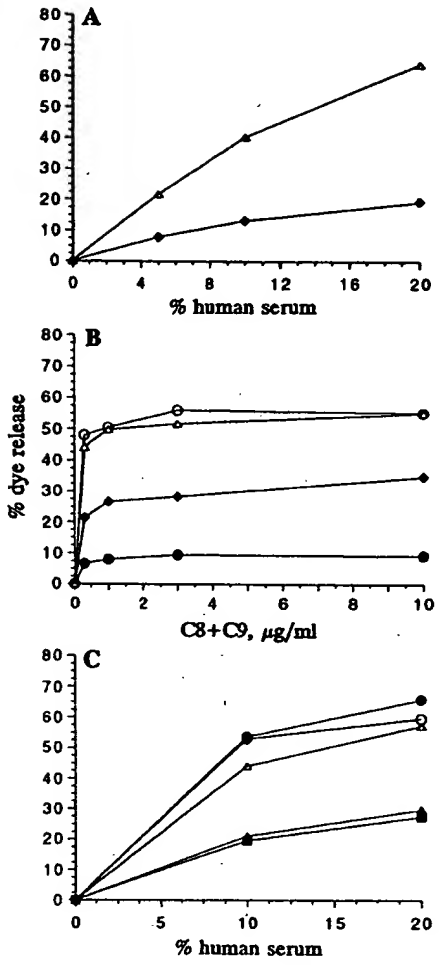

FIG. 5. Complement-mediated dye release assays on porcine PBMCs and cultured peripheral blood adherent cells. (*A*) Dye release assay performed on porcine PBMCs (◆), transgenic pig *H2K*^{b}–hCD59 153-2; (△), negative littermate control. (*B*) Dye release assay performed on PBMCs from transgenic pig *H2K*^{b}–hCD59 153-2 incubated in the presence of anti-hCD59 polyclonal antiserum (◆); control class I antibody PT85A (●); negative littermate control PBMCs incubated in the presence of anti-hCD59 polyclonal antiserum (△); control class I antibody PT85A (○). (*C*) Complement-mediated dye release assays on porcine peripheral blood adherent cells from pig *H2K*^{b}–hCD59 153-2; uninduced cells (△), PHA supernatants (■), PHA supernatants + hrTNF-α (▲), hrTNF-α (●), and control negative littermate cultured peripheral blood adherent cells (○).

level of hCD59 expressed on the cell surface, experiments were performed on the cultured monocyte lines derived from the *H2K*^{b}–hCD59 153-2 transgenic pig, which showed increased cell surface expression in response to cytokine treatments (see Fig. 2). Significantly, these monocytes demonstrated increased susceptibility to human complement-mediated lysis, consistent with the loss of hCD59 expression (Fig. 5*C*). As previously shown, culture of these cells in the presence of cytokines known to induce the MHC class I promoter—i.e., IFN-γ and TNF-α—upregulated hCD59 expression (Fig. 2*C*). Importantly, upregulating hCD59 expression restored their complement-resistant phenotype (Fig. 5*C*). These results confirm that the level of transgene expressi n correlates with cellular protecti n and also highlight the potential utility of the inducible *H2K*^{b} promoter in the setting of a cytokine-mediated inflammatory response.

DISCUSSION

Expression of human complement inhibitor hCD59 was established in transgenic mice and in a transgenic pig utilizing the murine MHC class I gene as a genomic expression cassette. The proteins encoded by the MHC class I genes from human (HLA), mouse (MHC), and swine (SLA) are expressed in most somatic cell types including the vascular endothelium (22, 24, 26). Therefore, a MHC class I promoter should direct high-level transgene expression in the endothelial cells of vascularized organs. The additional advantage to this genomic expression strategy is that the class I promoter has the capacity to upregulate hCD59 expression in response to the inflammatory cytokines IFN-γ and TNF-α (22, 24, 25).

We have approached the problem of complement-mediated hyperacute rejection during pig-to-primate xenotransplantation by engineering the xenogeneic donor tissue with human complement inhibitor hCD59. The analyses of hCD59 in *H2K*^{b}–hCD59 transgenic mice and transgenic pig 153-2 demonstrated that the *H2K*^{b}–hCD59 genomic construct regulated the expression of hCD59 in the context of a transgenic genome. Cell surface expression of hCD59 was detected in a variety of cells and tissues, including the vascular endothelium. The assays used to determine the protective effects of hCD59 expressed on the transgenic cells were performed with human whole serum, which contains serum complement components, as well as high-titer natural antibodies (W.L.F. and S.A.R., unpublished data). In addition, anti-porcine lymphocyte antiserum was used to enhance the activation of the classical complement pathway on the surface of the target cell. Our data demonstrated that the level of hCD59 expressed on the cell surface protected the xenogeneic cell even in the presence of additional complement-activating antibodies.

The utility of blocking complement as a method to prevent hyperacute rejection in pig-to-primate xenotransplantation was demonstrated by using cobra venom factor (CVF) and recombinant soluble complement receptor type 1 (sCR1) (refs. 27 and 28, respectively). A significant delay of complement-mediated hyperacute rejection in pig-to-primate heterotopic cardiac xenotransplantation was observed with the administration of CVF for two consecutive days before transplantation (27) or with a single intravenous bolus of sCR1 before xenograft reperfusion (28). The advantage of developing a transgenic donor animal expressing a human complement inhibitor is to provide the donor tissue with an endogenously expressed membrane-bound inhibitor and therefore does not rely on repeated administration of pharmacological agents.

The successful engineering of transgenic swine expressing a human complement inhibitor, and the demonstration that cells from these animals were significantly protected from human complement attack, suggests that this strategy may represent a useful component of an overall approach to discordant xenotransplantation. This transgenic approach will hopefully make porcine-to-primate transplantation models feasible that will allow the cellular aspects of discordant xenograft rejection to be evaluated. In addition, the production of porcine organs resistant to hyperacute rejection may open therapeutic windows for organ transplantation into humans, particularly when this technology is coupled with advances in cellular immunosuppressive regimens.

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Appendix E

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EXPRESSION OF HUMAN CD59 IN TRANSGENIC PIG ORGANS ENHANCES ORGAN SURVIVAL IN AN EX VIVO XENOGENEIC PERFUSION MODEL[1,2]

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The serious shortage of available donor organs for patients with end stage organ failure who are in need of solid organ transplantation has led to a heightened interest in xenotransplantation. The major barrier to successful discordant xenotransplantation is hyperacute rejection. Hyperacute rejection results from the deposition of preformed antibodies that activate complement on the luminal surface of the vascular endothelium, leading to vessel occlusion and graft failure within minutes to hours. Endogenous membrane-associated complement inhibitors normally protect endothelial cells from autologous complement—however, these molecules are species-restricted and therefore are ineffective at inhibiting activated xenogeneic complement. To address the pathogenesis of hyperacute rejection in the pig-to-human combination, F_1 offspring were generated from a transgenic founder animal that was engineered to express the human terminal complement inhibitor hCD59. High-level cell surface expression of hCD59 was detected in the hearts and kidneys of these transgenic F_1 animals, similar to expression levels in human kidney tissue. The hCD59 was expressed on both large vessel and capillary endothelium. Ex vivo perfusion experiments, using human blood as the perfusate, were performed with transgenic porcine hearts and kidneys to evaluate the ability of hCD59 to inhibit hyperacute rejection. These experiments demonstrated that transgenic organs expressing hCD59 resisted hyperacute rejection, as measured by increased organ function for both the hearts and the kidneys, as compared with control pig organs. Hearts from hCD59-expressing animals demonstrated a five-fold prolongation in function compared with controls, 109.8 ± 20.7 min versus 21.2 ± 2.9 min ($P = 0.164$). The hCD59-expressing kidneys also demonstrated significantly prolonged function at 157.8 ± 27.0 min compared with 60.0 ± 6.1 min for controls ($P = 0.0174$). Deposition of C9 neoantigen in the vasculature of porcine organs perfused with human blood was markedly reduced in organs expressing hCD59. These studies demonstrate that C5b-9 plays an important role in hyperacute rejection of a porcine organ perfused with human blood and suggest that donor pigs transgenic for hCD59 may be an integral component of successful clinical xenotransplantation.

The major barrier to successful discordant pig-to-primate xenogeneic organ transplantation is the phenomenon of hyperacute rejection (HAR)* (*1–5*). HAR results from the deposition of naturally occurring antibodies that activate complement on the luminal surface of the xenogeneic vascular endothelium. This antibody- and complement-mediated inflammatory response leads to endothelial cell destruction, vessel occlusion and graft failure within minutes after revascularization of the xenogeneic donor organ. Old World primates, including humans, have high levels of preexisting circulating antibodies that predominantly react with carbohydrate epitopes expressed on the surface of the xenogeneic cells from discordant species. Recent evidence indicates that most of these antibodies react with the carbohydrate epitope, Gal(α1,3)Gal (*6*), an epitope absent from Old World primates due to the lack of a functional α1,3 galactosyltransferase enzyme (*7*).

Several strategies aimed at blocking hyperacute rejection involve inhibiting complement activation or natural antibody reactivity (*8*). The utility of blocking complement as a method to prevent HAR in pig-to-primate xenotransplantation was demonstrated using cobra venom factor (CVF) and recombinant soluble complement receptor type 1 (sCR1) (*9*, *10*). A marked delay of HAR in pig-to-primate heterotopic cardiac xenotransplantation was observed with the administration of CVF for two consecutive days prior to transplantation (*9*) or with a single intravenous bolus of sCR1 prior to xenograft reperfusion (*10*). Additional studies demonstrated prolongation of cardiac function and prevention of HAR when a monoclonal antibody (mAb) to human C5 was added to human blood used to perfuse a porcine heart ex vivo (*11*). Methods used to address the efficacy of inhibiting natural antibody reactivity during hyperacute organ rejection include removal of xenoreactive antibodies (*12–14*) or inhibiting antibody binding using soluble carbohydrate therapy (*15*). An alterna-

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* Abbreviations: CVF, cobra venom factor; HAR, hyperacute rejection; hCD59, human CD59; mAb, monoclonal antibody; sCR1, soluble complement receptor type 1.

tive to these methods is to engineer don r organs with endogenous mechanisms to prevent antibody binding or complement activation (*16–18*).

Several groups have tested this strategy in vitro by engineering cultured porcine aortic endothelial cells to express human membrane-associated complement inhibitors on the endothelial cell surface (*19, 20*). These inhibitors effectively prevent activation of homologous complement but are unable to inhibit complement from divergent species (*21, 22*). Expressing human complement inhibitors on porcine endothelial cells provided these cells with a protective barrier against the cytotoxic effects of human complement (*19, 20*). To test the efficacy of a human complement inhibitor in a whole organ model, Fodor et al. developed transgenic pigs that expressed human CD59 (*16*). CD59 functions as a terminal complement inhibitor by preventing the binding of C9 to C5b-8, thus inhibiting formation of the membrane attack complex (*21*). Expression of hCD59 was detected on peripheral blood mononuclear cells as well as on vascular endothelial cell surfaces. In addition, expression of the hCD59 transgene significantly protected porcine endothelial cells from complement-mediated cytotoxicity in vitro and suggested that organs from this animal would be resistant to HAR in a pig-to-primate transplant model. In the current study, we demonstrate hCD59 RNA and protein expression in the h arts and kidneys of F_1 offspring derived from the transgenic founder animal. Immunohistochemical analysis of heart and kidney tissue revealed significant vascular expression of hCD59. The ability of hCD59 to prevent HAR was tested in an ex vivo porcine organ perfusion system using human blood as the perfusate. The results demonstrate that hCD59-expressing transgenic organs exhibited an increase in survival compared with normal pig organs.

MATERIALS AND METHODS

Generation of human CD59-expressing transgenic founder animals. Development of the transgenic founder animal has previously been described (*16*). The founder animal was bred to several gilts and F_1 hybrid offspring were screened for the hCD59 transgene by slot blot analysis from tail vein resections.

RNA preparation and RT-PCR analysis of hCD59 expression in transgenic organs. Tissues were harvested from euthanized animals and quick-frozen in liquid N_2. RNA isolation was performed using the acid-phenol Gu-isothiocyanate procedure. First strand cDNA was prepared by reverse transcription using Reverse Transcriptase (Seikagaku America, Inc. Rockville, MD) according to the manufacturer's specifications. Analysis of hCD59 expression was performed using the polymerase chain reaction and the following primers: (*1*) 5'-TGTCCTAA CCCAACTGCTGACTGCAAAACAGCCGTC; (*2*) 5'-GCGGTGACGAAATACCTCAGCG AGTGTGG. The PCR primer (*1*) is complementary to hCD59 and primer (*2*) is complementary to exon 2 of the H2Kb gene portion of the transgene. The PCR product expected from a correctly spliced mRNA is 425bp, whereas genomic DNA or an unprocessed messenger RNA will yield a product 575bp. The β-actin primers (*1*) 5'CCAACTGGGACGACATGGAG; and (*2*) 5'-AGGTCCAGACGCAGGATGGC were used as a positive control for each tissue RNA, and produce a 300bp PCR product from a correctly processed β-actin mRNA. All PCR reactions were performed using the following reaction conditions: 5 μl of 1st strand cDNA reaction, 2.5 pmol of each primer, 50 mM KCl, 10 mM Tris-HCL pH 8.3, 2 mM $MgCl_2$, 0.4 mM dNTPs (dGTP, dCTP, dATP, dTTP), 2 U of Taq Polymerase (Perkin-Elmer, Cetus), in a 100 μl final volume. The following temperature conditions were used for the hCD59 PCR reactions: 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The β-actin PCR reactions were performed as described for hCD59, except that a 50°C annealing temperature was used. The hCD59 PCR reactions were analyzed by electrophoresing 20 μl of a 100 μl reaction on a 1 × TBE 1% agarose gel + 1 μg/ml ethidium bromide. Control PCR reactions used to detect hCD59 expression from human tissue were performed on human liver first-strand cDNA. Total human liver RNA (Clonetech, Palo Alto, CA) was used as a template to produce first-strand cDNA as described above. PCR detection of hCD59 expression was determined using the reaction conditions as described above for the detection of hCD59 in the transgenic pig tissue, with the exception of using the following hCD59 5' and 3' untranslated region specific primers: 5' primer, 5'-GGAAGAGGATCCTGGGCGCCGCAGG; 3' primer, 5'-CCCAACAG-GATCCATTTGGAAAATATCAAGCC. The expected reaction product from a correctly spliced hCD59 mRNA equals 535bp. The β-actin controls and agarose gel analysis of the reactions products were performed as described above. Then 15 μl of the 100 μl β-actin PCR reaction was analyzed as described for hCD59.

Animals and perfusion circuits. Outbred swine obtained locally weighing ~20 kg served as organ donors for control studies. F_1 offspring of similar size expressing the hCD59 transgene were used in the experimental group. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals," (National Institutes of Health publication 85–23, 1985 revision). All procedures were approved by the University of Minnesota Research Animal Resources Committee on Animal Care and Use. Animals selected for study were anesthetized with sodium pentothal and placed on a ventilator for maintenance anesthesia with isofluorane and oxygen. Following systemic heparinization, hearts were procured after topical cold-induced cardiac arrest. Kidneys were then procured and flushed with cold EuroCollins's preservation solution and stored at 4°C until use.

Hearts were prepared and perfused ex vivo as previously described (*14*). Hearts were perfused with one unit of fresh human blood from normal donors. Each unit of human blood (450 ml) was anticoagulated with 1.2 units heparin/ml and used within 3 hr. Functional parameters measured included heart rate and rhythm, ECG R wave voltage, oxygen consumption, and coronary artery resistance. Blood samples were obtained at defined time points for measurement of antibody and complement and complete blood counts, including hemoglobin, platelet, and neutrophil counts.

Kidneys were perfused ex vivo 24 to 48 hr following procurement. Kidneys were perfused via the renal artery at a constant pressure of 60–80 mmHg. Flow was continuously monitored. Functional parameters measured included renal vascular resistance, oxygen consumption, and urine output. Renal perfusion circuits were primed with the addition of 450 ml blood from a single donor and 100 ml crystalloid solution consisting of Ringer's lactate with the addition of glucose, insulin, calcium, and furosemide. Blood samples were obtained prior to priming the perfusion circuit (baseline), at circuit priming, at 15 min intervals during perfusion for the first hour and then at hourly intervals for antibody, complement and hematology profile measurements.

Antipig endothelial cell IgG and IgM antibody levels. Antipig endothelial cell IgG and IgM antibody levels were determined using an ELISA (*23*). Pig aortic endothelial cells were explanted and cultured in 96- well plates in monolayer and fixed with 0.1% glutaraldehyde. After incubation with HBSS-25 mM HEPES, pH 7.4 (Gibco, Grand Island, NY), containing 1% bovine serum albumin to block nonspecific binding sites, dilutions of human serum samples were added to wells, followed by washing. Then goat antihuman IgG or IgM conjugated to alkaline phosphatase was added, and these were incubated for 60 min at room temperature or 4°C, respectively. The plates were then developed with a solution consisting of 1 mg/ml p-nitrophenyl phosphate and 100 mM diethanolamine in 0.5 mM MgCl2, pH 9.5. Absorbance at 405 nm was determined with a V_{max} kinetic plate reader (Molecular Devices, Sunnyvale, CA). Negative controls were prepared as above, but application of human serum was omitted.

LISA units were calculated at 1:2 dilutions by comparison with a rum pool consisting of normal human blood donors; the A_{405} of the ıoled source at 1:2 dilution was used as 100 units. Total IgG and M concentrations were determined by nephelometry.

CH50 measurements. Hemolytic complement activity of the clas-cal pathway (CH50) was measured in 96-well flat-bottomed micro-ter plates (Corning, Corning, NY). Frozen serum samples were ıawed and serially diluted two-fold from undiluted serum or from n initial 1:10 dilution in a buffer consisting of 5 mM Na Veronal, 145 ıM KCl, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$, containing 0.5% human erum albumin (VB). Then 50 μl of diluted serum was mixed with 50 ıl of sensitized sheep red cells at a concentration of 10^8 red cells/ml n VB (*24*). Finally, 150 μl of VB was added and the plates were ncubated at 37°C with occasional mixing. Degree of hemolysis was neasured by turbidity at 650 nm (*25*) in a V_{max} kinetic microplate eader (Molecular Devices). CH50 was expressed as the serum dilu-ion yielding 50% reduction in turbidity.

Immunohistology. Endomyocardial biopsy specimens were ob-tained from the right ventricle via the superior vena caval–right atrial opening following harvest of the heart just before reperfusion, and then at the completion of each experiment. Tissue specimens were formalin-fixed, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Tissues were also embedded in OCT gel and snap-frozen in precooled isopen-tane and stored at −80°C until used. Cryostat-prepared tissue spec-imens were stained with FITC-conjugated goat antibodies specific for IgG (Kallestad Diagnostics, Chaska, MN); IgM, C3, C4, and fibrino-gen (Organon Teknika Corporation, Durham, NC); and C1q and properdin (INCStar Corp., Stillwater, MN). Antibodies were preab-sorbed with pig serum prior to use to eliminate nonspecific binding. In addition, tissues were stained with a murine antihuman mAb specific for C9 neoantigen (*26*), followed by a fluorescein-labeled goat antimurine mAb (Organon Teknika Corp.). Tissues were also stained pre- and postperfusion with a murine antihuman CD59 mAb MEM43 (Biodesign International, Kennebunkport, ME).

RESULTS

Analysis of CD59 expression. Preliminary characterization of the F_1-positive animals indicated that the level of expres-sion on p ripheral blood mononuclear cells was comparable to the expression observed in the founder animal (unpub-lished data). Transgenic F_1 animals were further analyzed for hCD59 expression utilizing a reverse transcription PCR (RT-PCR) approach, to ensure that the organs were xpress-ing the transgene prior to th perfusion experiments. RNA isolated from various sections of the heart and kidney were used to isolate mRNA. RT-PCR analysis of those mRNA preparations revealed an hCD59- specific DNA fragment that was only present in the transgenic tissue (Fig. 1A). Human CD59 was detected in both atrial and ventricle sec-tions of the transgenic heart. The coronary arteries also exhibited hCD59 expression (Fig. 1A). Expression of hCD59 in the kidney was detected in the medulla as well as in the kidney cortex (Fig. 1A). The transgenic negative littermate control animal showed no hCD59 expression (Fig. 1A). A control PCR performed on a human liver cDNA preparation to demonstrate the levels of hCD59 expression in a control human tissue revealed a 535bp fragment that is consistent with a correctly spliced hCD59 transcript (Fig. 1C). A control β-actin PCR reaction was performed on all tissues examined to demonstrate comparable quantity and quality of the first-strand cDNA derived from the tissue RNA. All tissue sam-ples revealed a 300bp β-actin-specific DNA fragment that represents a correctly processed mRNA (Fig. 1, B and C).

The RT-PCR data confirmed hCD59 expression in the transgenic organs and revealed that various sections from either the heart or the kidney exhibited consistent expression patterns—however, this analysis cannot discriminate cell type–specific expression. To localize the cellular distribution of CD59 expression, immunohistochemical analysis was per-formed on heart and kidney organ sections with the hCD59-specific monoclonal antibody, MEM43. The expression of hCD59 in the heart tissue was exclusive to the vasculature, whereas the striated cardiac muscle was devoid of any hCD59 expression (Fig. 2A). Immunohistochemical analysis of the kidney revealed similar expression in the vascular endothelium with intense expression in glomerular struc-tures (Fig. 2B). Tissues from normal porcine hearts and kid-neys showed no expression of hCD59 (negative control). Hu-

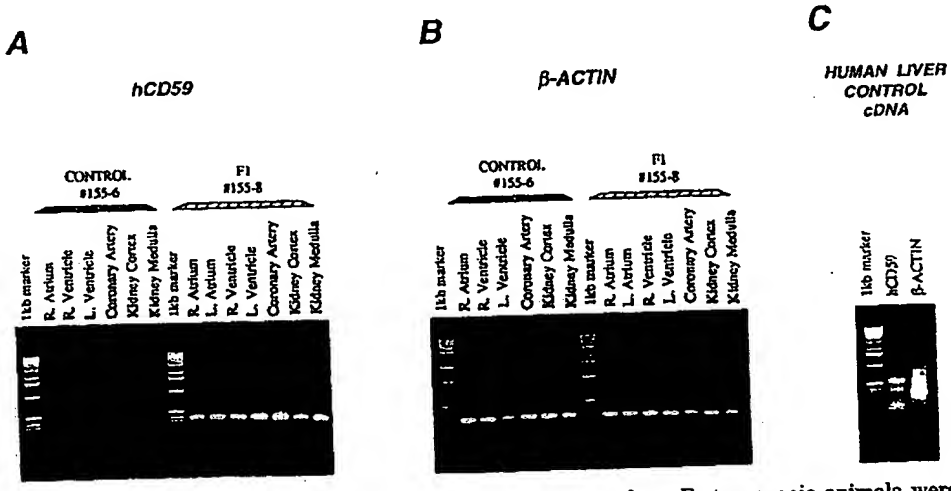

FIGURE 1. RT-PCR analysis of hCD59 expression. (A) Cardiac and renal tissue from F_1 transgenic animals were analyzed for hCD59 expression using an RT-PCR approach. Analysis of mRNA preparations detected hCD59 expression in atrial and ventricular tissues as well as in the coronary arteries. Expression of hCD59 was also detected in the renal cortex and renal medulla. Transgenic negative littermate control PCR reactions showed no expression of hCD59. (B) All tissue samples were tested for the expression of a control β-actin mRNA and revealed a 300bp β-actin-specific DNA fragment that corresponds to a correctly processed mRNA. (C) Human liver cDNA was used as a control human tissue and revealed the level of CD59 expression from a representative human tissue control.

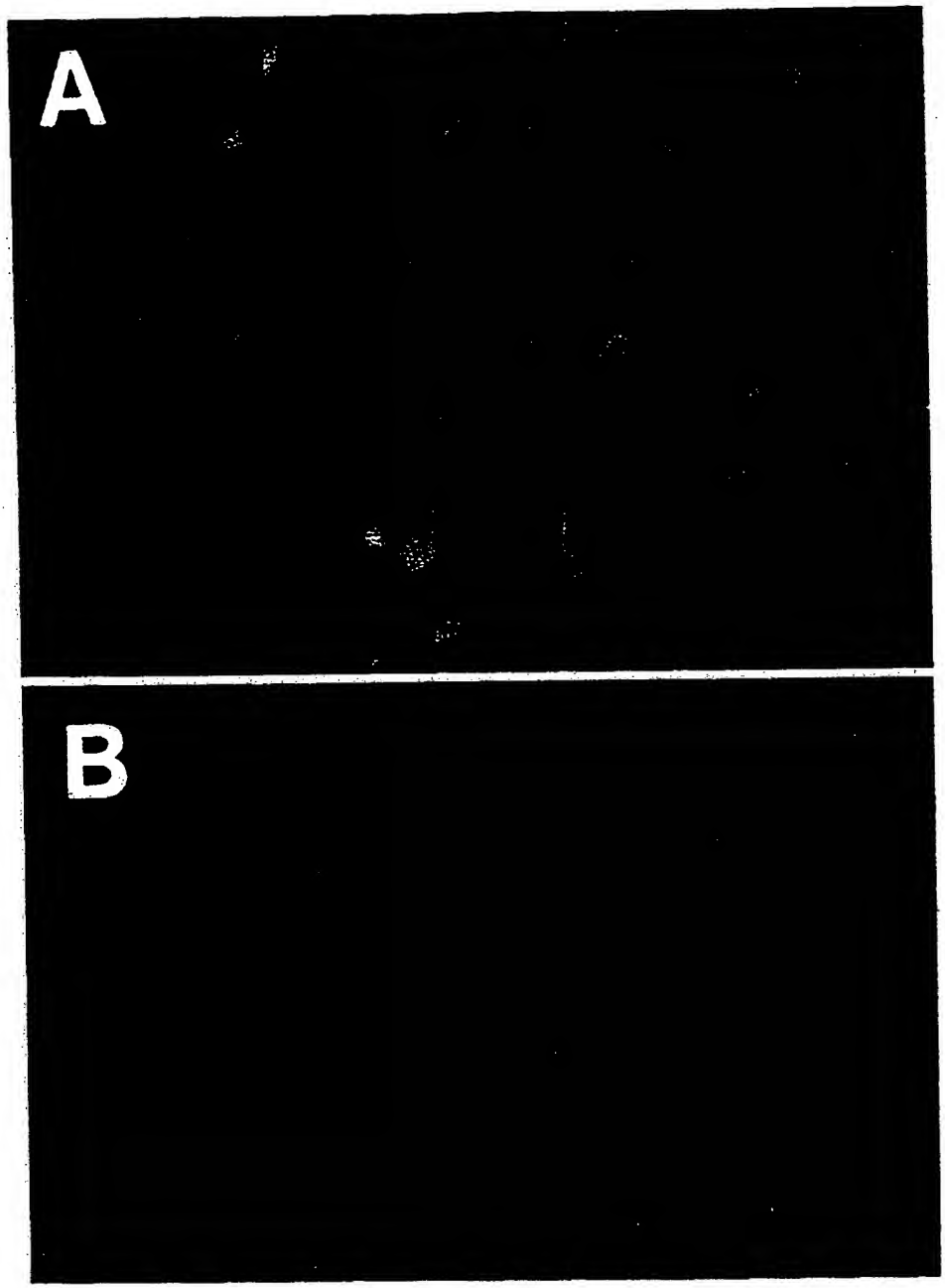

FIGURE 2. Immunohistochemical analysis of heart and kidney tissues prior to perfusion. Representative right ventricular endomyocardial and renal coxtex tissue samples were obtained prior to perfsuion with human blood and examined for the presence of hCD59. Shown are heart tissue [panel A] and kidney tissue [panel B] with heavy levels of expression of hCD59.

man kidney tissue was used as a positive control for the anti-hCD59 mAb.

Effect of hCD59 expression on cardiac function. To determine whether expression of hCD59 in porcine tissue would protect against HAR, F_1 offspring expressing the hCD59 transgene underwent procurement of their hearts and perfusion with human blood. In these experiments, the time to cessation of normal sinus cardiac rhythm (heart survival time) was the endpoint used for each perfusion experiment. In establishing this model, porcine hearts perfused ex vivo with autologous blood demonstrated normal sinus cardiac rhythm and stable functional parameters for a 6 hr study interval (negative controls; data not shown). Normal porcine hearts perfused ex vivo with human blood resulted in HAR at 21.2 ± 2.9 min (n = 5; positive controls). In these experiments, coronary vascular resistance markedly rose until cessation of normal sinus rhythm, with high level of oxygen consumption and rapid decline in R wave voltage.

One negative littermate for the hCD59 transgene underwent procurement and perfusion of its heart with human blood and demonstrated an atrioventricular dissociative rhythm for 58 min (complete heart block), but did episodically demonstrate normal rhythm for brief periods. Coronary vascular resistance was markedly elevated immediately upon perfusion with human blood, being 4 to 5 times that of the levels of hCD59 pig hearts, and continued to rise throughout the entire experiment. This heart also showed a marked utilization of oxygen, but to a lesser degree than hCD59

arts. On visual inspection, this heart appeared to function orly and demonstrated gross morphologic changes consist with HAR, turning dark purpl , with petechial hemorages on the epicardial surface.

Four hCD59-expressing porcine hearts underwent perfuon with human blood. Survival time varied from 63 to 163 in, with a mean of 109.8 ± 20.7 min ($P = 0.0146$ compared ith positive controls; Fig. 3A). All hCD59 porcine hearts monstrated slowly rising coronary vascular resistance unl 15 to 30 min prior to cessation of normal sinus rhythm, at hich time a dramatic rise in vascular resistance was noted iat was 2 to 20 times as high as measurements in the first 5 to 30 min of perfusion. Each hCD59-expressing pig heart lso showed marked oxygen consumption throughout perfuion with an increase in oxygen extraction during the last 15 30 min of perfusion that correlated with the increase in oronary artery resistance. This was attributed to the derease in oxygen delivery resulting from the decrease in blood low toward the end of each experiment. R wave voltage esponded in a similar manner, slowly rising for the first 30 nin of perfusion and then gradually declining the last 30 to 0 min of each experiment. Hearts functioning for over 100 nin showed stabilization of coronary vascular resistance and voltage measurements for the intervening period after 30 min and prior to the last 30 to 45 min of perfusion. These hearts demonstrated a significant increase in survival time compared with normal pig hearts perfused with human blood, and on visual inspection appeared to function normally for most of the perfusion interval.

Effect of hCD59 expression on renal function. Kidneys were also harvested from pigs expressing the hCD59 transgene and perfused with unmodified human blood. Each renal perfusion experiment was terminated when thrombosis occurred or electively at 4 hr of perfusion. In establishing negative controls, pig kidneys perfused with fresh autologous blood demonstrated slowly rising renal vascular resistance and produced measurable urine output for 4 hr (data not shown). Renal vascular resistance gradually rose to 3 times initial levels by 4 hr of perfusion. Oxygen consumption was also noted throughout the perfusion interval and increased in parallel with the increase in renal vascular resistance.

Four kidney perfusion experiments were performed with normal porcine kidneys perfused with human blood (positive control). Negative control experiments were performed by perfusion of normal porcine kidneys with autologous blood (data not shown). Two kidneys from an outbred pig underwent perfusion with human blood. These 2 kidneys functioned for 60 min and 45 min, respectively. Each demonstrated substantial urine production for 30 min, which then abruptly dropped to zero. Renal vascular resistance was markedly elevated during each study. Two kidneys from a negative littermate for the hCD59 transgene also underwent perfusion with human blood. The first kidney demonstrated elevated renal vascular resistance measurements throughout perfusion. Urine output was vigorous for the first 60 min of perfusion, then abruptly declined to zero just prior to rejection at 75 min. The second kidney had markedly elevated renal vascular resistance measurements and had no measurable urine output for the entire study. This kidney had gross morphological evidence of thrombosis by 1 hr. Survival time was estimated as 1 hr from this experiment. Mean survival for these 4 experiments was 60.0 ± 6.1 min (SEM).

Seven hCD59-expressing transgenic pig kidneys underwent perfusion with human blood. Survival time for these kidneys ranged from 70 to 240 min (mean of 157.8 ± 27.0) compared with 60.0 ± 6.1 min for normal porcine kidneys perfused with human blood ($P = 0.0174$; Fig. 3B). Renal vascular resistance was elevated in all hCD59 porcine kidneys compared with normal pig kidneys perfused with human blood but showed variation in subsequent vascular resistance readings. The 7 hCD59-expressing porcine kidneys that were perfused with human blood demonstrated two distinct survival patterns. Four kidneys demonstrated function for over 3.5 hr and showed a gradual decrease in vascular resistance for the first 1.5 to 2 hr of perfusion, followed by an increase in vascular resistance until completion of each experiment. These findings were similar to the changes in vascular resistance seen in n rmal pig kidneys perfused with fresh autologous blood. Thre additional hCD59-expressing pig kidneys underwent perfusion and functioned for 70, 75, and 105 min. Each of these kidneys had renal vascular resistance measurements that were either profoundly levated at the initiation of perfusion or rose rapidly during perfusion,

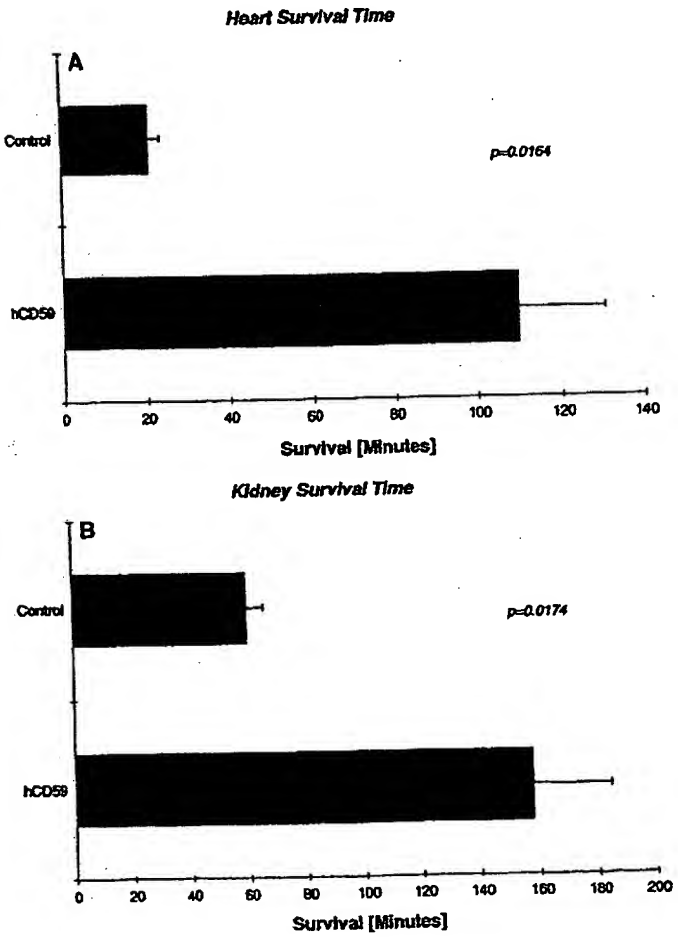

FIGURE 3. Organ survival time. Time to completion of each organ perfusion experiment is summarized. Time to cessation of heart function [panel A] is shown for control heart perfusion experiments ($n = 5$) and for hCD59 hearts ($n = 4$). Time to completion of kidney perfusion experiments [panel B], defined by organ thrombosis, is also shown for control experiments ($n = 4$) and for hCD59 kidneys ($n = 7$).

similar to vascular resistance changes seen in positive control perfusion experiments.

Antiporcine endothelial cell natural antibody levels. All human blood donors used in this study were screened and selected based on normal levels of xenoreactive natural antibodies prior to ex vivo perfusion of hCD59-expressing organs. No hCD59 organ perfusion study was compromised by low xenoreactive IgM levels. Human antiporcine natural antibody levels were measured for each donor immediately prior to organ perfusion (baseline) and at defined time points throughout each perfusion experiment. Blood donors used to perfuse hCD59-expressing porcine hearts had similar levels of xenoreactive IgM ($P = 0.77$) and IgG ($P = 0.15$) compared with donors used to perfuse normal pig hearts (Fig. 4). Xenoreactive antibody levels decreased during organ perfusion, with minimal levels of natural antibody detected following 30 min of perfusion. Several hCD59-expressing porcine organ perfusion studies were performed with repeat donors whose blood had been used in control studies several months previously. Perfusion of one hCD59-expressing pig heart with blood from a repeat donor resulted in a 6-fold prolongation of organ survival—163 min compared with 28 min—when blood from this donor was used to perfuse a normal pig heart in a study performed several months earlier. In addition, perfusion of hCD59 porcine kidneys was performed with previously used donors for control heart and kidney organ perfusion experiments. These blood donors all demonstrated high xenoreactive nAb levels.

Total complement hemolytic activity. Total complement hemolytic activity was determined at baseline levels as well as at specified time points during the perfusion interval. All blood donors demonstrated normal complement levels as measured by CH50 assay. In comparison with normal hearts, hCD59-expressing hearts perfused with human blood elicited substantially less activation of complement during perfusion. CH50 measurements were not different at baseline (73.3 ± 2.6 for hCD59 hearts vs. 75.1 ± 1.9 for normal hearts; $P = 0.77$) but were markedly different at 30 min of perfusion (42.2 ± 3.2 vs. 22.5 ± 3.7; $P = 0.02$) (Fig. 5). This attenuation in complement activation persisted throughout perfusion of each CD59 heart.

Kidneys perfused with human blood showed similar findings, with a decrease in complement activation in hCD59-expressing porcine kidneys compared with normal kidneys. Thus the presence of the terminal complement inhibitor on the vascular endothelium resulted in substantially less activation of complement in both hearts and kidneys.

Histopathology and Immunopathology. Porcine organs perfused with human blood were studied for the presence of immunoglobulin and complement deposition as well as the expression of hCD59. Histologic analysis of control heart and kidney tissues obtained at the end of each perfusion study showed characteristic changes consistent with HAR, including marked interstitial hemorrhage and edema, neutrophil infiltration, detachment of endothelial cells from the basement membrane, and microvascular thrombosis. The hCD59-expressing organs showed the same changes in a majority of

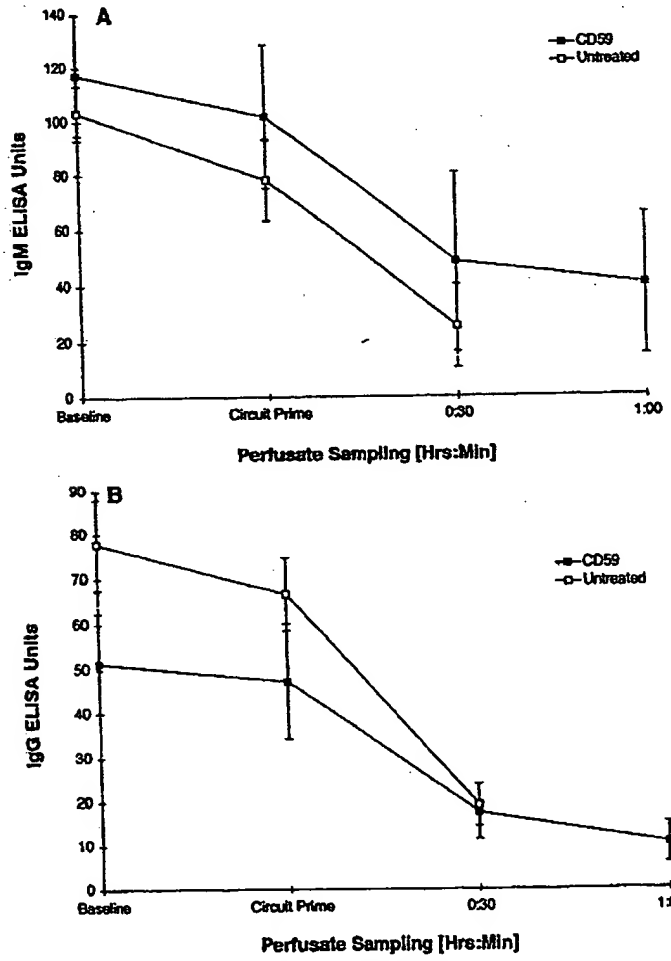

FIGURE 4. Antiporcine natural antibody levels. Plasma endothelial cell IgM [panel A] and IgG [panel B] levels at baseline, following priming of the perfusion circuit (circuit prime), and at specified intervals following perfusion of a porcine heart. Data are expressed as mean values ± SEM for hCD59 hearts compared with control hearts.

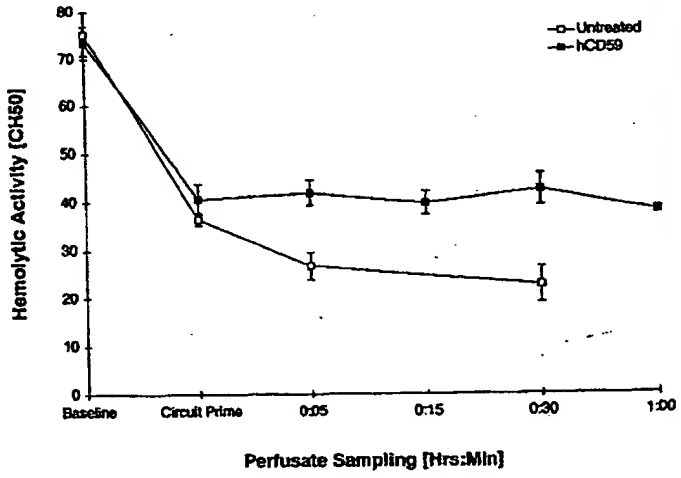

FIGURE 5. CH50 levels. Total complement hemolytic ctivity [CH50] in human plasma is shown for heart perfusion experiments before (baseline) and after priming the perfusion circuit (circuit prime) as well as at specified intervals for hCD59-expressing hearts ($n = 4$) compared with control hearts ($n = 5$). CH50 were not significantly different at baseline ($P = 0.77$) but were significantly different by 30 min of perfusion ($P = 0.02$), indicating a lesser degree of complement activation by hCD59-expressing porcine hearts.

e organs studies but were more focal and widely distrib- ed. Two hCD59-expressing kidneys that were perfused for hr demonstrated relatively normal tissue architecture.

Nontransgenic control porcine hearts and kidneys perfused ith human blood demonstrated mark d deposition of IgM, ;G, C1q, C3, C4, and C9 neoantigen as well as moderate eposition of properdin. Similar levels of IgM, IgG, C1q, C3, 4, and properdin were deposited in hCD59-expressing por- ne organs (Table 1). The level of C9 neoantigen deposition as minimal in hCD59-expressing transgenic organs com- ared with normal porcine organs (Fig. 6). Thus, the presence f the hCD59 terminal complement regulatory protein re- ulted in a decrease in C9 neoantigen deposition and prolon- ation of organ function but was not able to prevent HAR rom eventually occurring.

DISCUSSION

Complem nt is known to play a critical role in the patho- enesis of HAR of discordant xenografts (*8*). HAR can be nhibited by agents that interfere with complement activa- ion (*8*). For example, in the pig-to-primate combination, urvival f a porcine heart was prolonged from minutes to 2–3 days by inactivating complement in the recipient with CVF (*9*). Prolongation of a cardiac xenograft was also achieved by administration of sCR1 (*10*). These agents may be useful in a clinical setting to inhibit complement at the time of transplantation and for a few days thereafter. How- ever, th se agents are clinically unattractive for continuous use because complement inactivation in the recipient may compromise the ability of the recipient to handle immune complexes and infectious processes, particularly if generation of the opsonic fragment C3b is impaired in immunosup- pressed patients. It would therefore be desirable to inhibit complement activation at the site of the xenograft itself and thus avoid interfering with host complement. For this reason, Dalmasso t al. proposed that inhibiting complement locally may be achieved by expressing membrane-associated com- plement inhibitors on the vascular endothelium of the graft that are from the same species as the recipient (*19*). Comple- m nt inhibitors such as DAF, membrane cofactor protein, and CD59 inhibit autologous complement but are usually ineffective in inhibiting complement of a different species. If a human complement inhibitor is expressed on the endothe- lium of a porcine organ, that inhibitor would then protect the porcine organ from attack by human complement. This hy- pothesis was tested first with purified human DAF that was incorporated into pig endothelial cells in vitro; when these cells wer exposed to human natural antibodies and comple- ment, the endothelial cells did not undergo complement-mediated cytotoxicity (*19*). Subsequently it was shown that human complement inhibitors that were transduced in xeno- geneic cells were able to inhibit killing of those cells by human complement (*20*). More recently transgenic mice and pigs were developed that expressed high levels f comple- ment inhibitors on their vascular endothelial cells (*16–18*). Prolonged survival of a porcine heart expressing hDAF and hCD59 heterotopically transplanted into a baboon was dem- onstrated (*18*).

In this study, we tested the ability of hCD59 expressed in transgenic pigs to protect organs against HAR when perfused ex vivo with human blood. First we showed that hCD59 is expressed in the vasculature of the hearts and kidneys of F_1 offspring derived from a transgenic founder animal. Cell surface expression of hCD59 was also detected in the vascu- lar endothelium of several solid organs, including the heart and kidney, as well as in aortic endothelial cells grown in culture (data not shown). When pig hearts and kidneys were perfused ex vivo with human blood, organ survival was pro- longed several-fold in comparison with controls not express- ing hCD59. Furthermore, two hCD59-expressing kidneys demonstrated normal function for the entire 4 hr study pe- riod. Immunohistochemical analysis of these organs demon- strated vascular deposition of IgM, IgG, C1q, C3, and C4 of an intensity similar to that in organs from control animals. In contrast, hCD59 transgenic hearts and kidneys perfused with human blood showed only minimal deposition of C9 neoantigen, whereas controls demonstrated intense deposi- tion of C9 neoantigen. CD59 inhibits complement-mediated membrane damage by preventing the binding of C9 to C5b-8 (*21*) and these experiments clearly demonstrate the role of C9 as a part of the membrane attack complex in elicitation of HAR. The experiments also suggest a potential for hCD59 expressed in transgenic donor organs to protect a xe- nografted organ from complement-mediated injury.

Several observations suggest that strategies to inhibit as- sembly of the membrane attack complex may be important in efforts to abrogate HAR. First, in vitro studies have demon- strated that the membrane attack complex is able to mediate activation of endothelial cells and platelets (*21*). Second, in vivo studies have shown cardiac graft survivals in C6-defi- cient rats and CVF-treated rats of 2–3 days and 3–4 days, respectively (*9*, *27*), compared with minutes in controls. Fi- nally, in our ex vivo perfusion model of pig-to-human xeno- transplantation, survival of porcine hearts was extended from a few minutes to more than 4 hr when C5b-9 deposition was blocked with a mAb against human C5 added to human blood prior to perfusing a porcine heart (*11*). Addition of this mAb resulted in a dose-dependent inhibition of organ tissue damage, suggesting that preventing formation of the C5b-9 complex would offer protection against HAR. The hCD59 functions in a similar manner, preventing assembly of the C5b-9 complex, implying that increased expression of hCD59 could confer significant protection from HAR. It has been shown before in vitro that hCD59 transduced in porcine endothelial cells protects these cells against human comple- ment (*20*). Now w demonstrate that transgenically ex- pressed hCD59 on porcine cardiac and renal vascular endo- thelium is able to provide significant protection against HAR in an ex viv organ perfusion model.

It is known that CD59 plays a dominant role in protecting human endothelial cells from cytotoxicity by autologous se- rum in comparison with DAF and membrane cofactor protein

TABLE 1. Immunopathologic tissue analysis

	hCD59 Hearts	Control hearts	hCD59 Kidneys	Control kidneys
IgM	+++	+++	+++	+++
IgG	+++	+++	+++	+++
C1q	+++	+++	+++	+++
C3	+++	+++	+++	+++
C4	+++	+++	+++	+++
C9	+/–	+++	+/–	+++
Properdin	++	++	++	++
hCD59	+++	–	+++	–

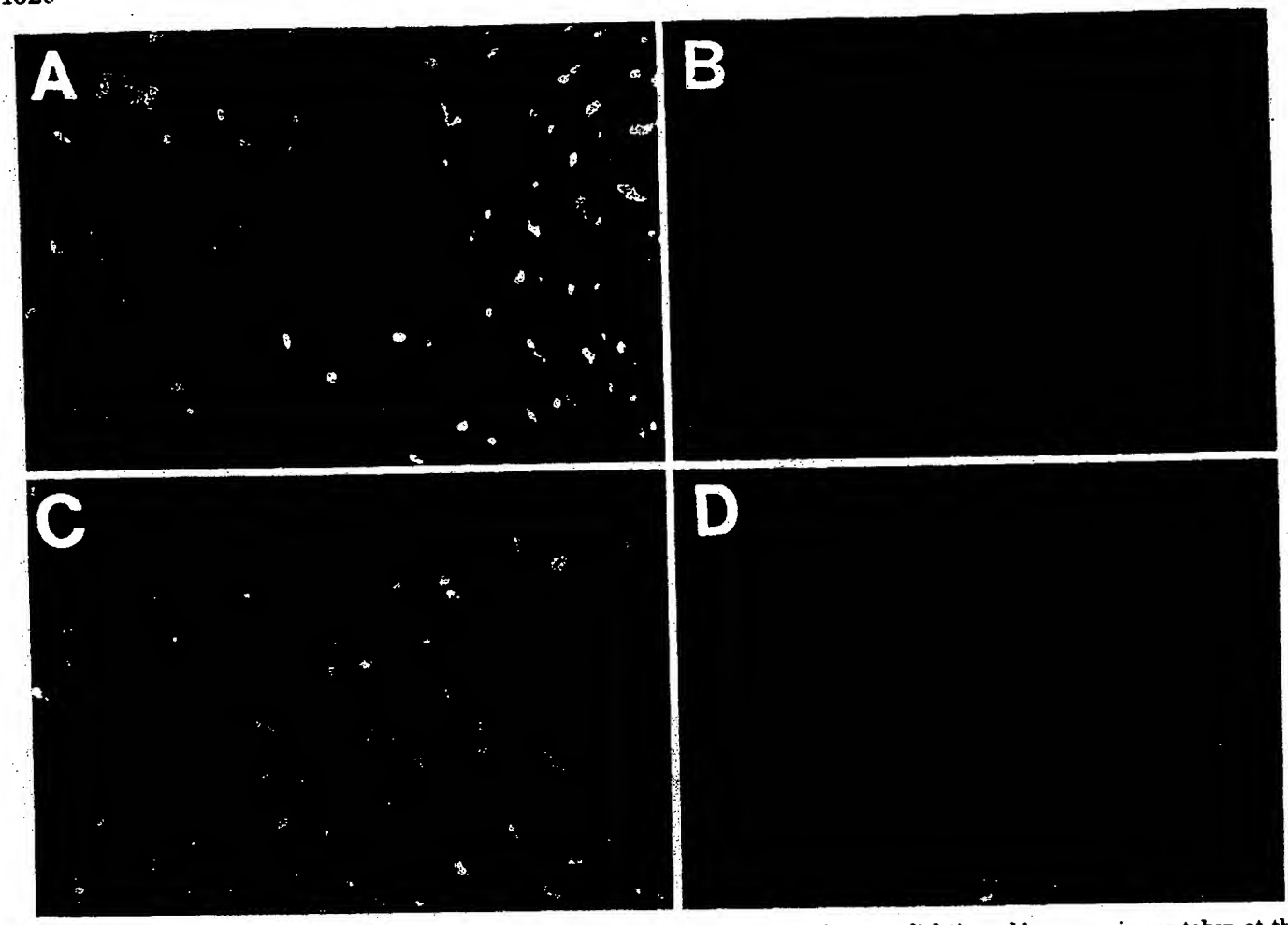

FIGURE 6. Immunohistochemical tissue analysis. Representative right ventricular endomyocardial tissue biopsy specimens taken at the completion of each perfusion experiment and examined for the deposition of C3 and C9. Shown are C3 deposition from control [panel A] and hCD59-expressing [panel B] heart tissue specimens as well as C9 deposition from control [panel C] and hCD59-expressing [panel D] hearts.

(*28*). CD59 also protects endothelial cells against activation processes that are mediated by C5b-9 (*29, 30*). It is likely, then, that hCD59 expression may be a major element in a strategy to prevent HAR in the pig-to-human combination. We found in this ex vivo perfusion model that hCD59 expressed on small and large vessels in porcine organs perfused with human blood induced prolongation of organ survival. However, expression of hCD59 in pig organs was insufficient to completely prevent tissue injury and organ thrombosis, which eventually occurred in most perfusion experiments.

These studies highlight the role of C9 in HAR and the potential usefulness of using transgenic donors that express the membrane attack complex inhibitor CD59. The use of donor pigs that express hCD59, together with human DAF and membrane cofactor protein, complement inhibitors that act at early stages of complement activation, would obviously be advantageous. This approach in combination with other methods such as selective removal of IgM anti-endothelial cell natural antibodies and immunosuppression may be useful in developing procedures that abrogate HAR.

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SHORT COMMUNICATION

Synthesis and secretion of the mouse whey acidic protein in transgenic sheep

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The synthesis of foreign proteins can be targeted to the mammary gland of transgenic animals, thus permitting commercial purification of otherwise unavailable proteins from milk. Genetic regulatory elements from the mouse whey acidic protein (WAP) gene have been used successfully to direct expression of transgenes to the mammary gland of mice, goats and pigs. To extend the practical usefulness of WAP promoter-driven fusion genes and further characterize WAP expression in heterologous species, we introduced a 6.8 kb DNA fragment containing the genomic form of the mouse WAP gene into sheep zygotes. Two lines of transgenic sheep were produced. The transgene was expressed in mammary tissue of both lines and intact WAP was secreted into milk at concentrations estimated to range from 100 to 500 mg/litre. Ectopic WAP gene expression was found in salivary gland, spleen, liver, lung, heart muscle, kidney and bone marrow of one founder ewe. WAP RNA was not detected in skeletal muscle and intestine. These data suggest that unlike pigs, sheep may possess nuclear factors in a variety of tissues that interact with WAP regulatory sequences. Though the data presented are based on only two lines, these findings suggest WAP regulatory sequences may not be suitable as control elements for transgenes in sheep bioreactors.

Keywords: whey acidic protein gene; transgenic sheep; bioreactor; mammary gland

Introduction

Whey acidic protein (WAP) is an abundant milk protein in mice, rats, rabbits and camels, but has not been found in pigs, sheep or cattle (for review see McKnight *et al.*, 1991). The WAP gene has been isolated from mice, rats and rabbits, and molecular mechanisms of its regulation are being analysed in transgenic animals. In mice, expression of the WAP gene is confined to mammary alveoli, and WAP RNA accumulates at the end of pregnancy. Steady-state levels remain high throughout lactation (Pittius *et al.*, 1988; Hennighausen *et al.*, 1991), with approximately 10% of the mRNA in lactating mammary tissue encoding WAP (Hennighausen and Sippel, 1982a,b). Because of its high-level tissue-specific expression, the WAP promoter has been used to direct expression of heterologous genes to alveolar epithelial cells for basic biological studies (Andres *et al.*, 1987; Schonenberger *et al.*, 1988; Jhappan *et al.*, 1993; Tzeng *et al.*, 1993) and to direct the synthesis of pharmacologically active human proteins to the mammary glands of goats and pigs, in the hope of producing commercial quantities of purified proteins (Ebert *et al.*, 1991; Velander *et al.*, 1993).

Although the mouse is frequently used to analyse the integrity and functionality of hybrid genes, ultimately commercial production of foreign proteins in milk will require the use of transgenic rabbits, pigs, sheep, goats or cows. This, however, requires that regulatory elements from mammary-gland-specific genes are functional across

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species boundaries. Towards this end, we compared the expression of WAP transgenes in mice (Burdon *et al.*, 1991a) and pigs (Shamay *et al.*, 1991; Wall *et al.*, 1991). Whereas only about one half of the mouse lines expressed the WAP transgene (Burdon *et al.*, 1991a), the WAP gene was expressed in all lines of pigs and the WAP was secreted into the milk (Shamay *et al.*, 1991; Wall *et al.*, 1991).

To extend the range of our findings, we report here a study designed to test the feasibility of using genetic regulatory elements from the mouse WAP gene to target gene expression to the mammary glands of sheep.

Material and methods

Generation of transgenic sheep

Ova, zygotes and 2-cell embryos (referred to hereafter as "eggs") were harvested from superovulated parous Rambouillet ewes as previously described (Rexroad *et al.*, 1990). The eggs were transferred to a test tube containing 37 °C Dulbecco's PBS with 10% sheep serum and transported to the laboratory for gene injection. In the laboratory, the eggs were microinjected with a 6.8 kb *Eco* RI fragment isolated from mouse genomic DNA (Campbell *et al.*, 1984) containing 2.6 kb 5′ and 1.6 kb 3′ flanking sequences surrounding the entire WAP gene. After microinjections, surviving eggs were returned to the surgical facility and two to four injected eggs were transferred into oviducts of anaesthetized recipient ewes whose cycles had been regulated with progestin pessaries.

Indentification of transgenic sheep

To identify transgenic sheep, genomic DNA was isolated from tail tissue and analysed for the presence of the mouse WAP gene using PCR and Southern blot analysis by the same procedure previously reported (Shamay *et al.*, 1991).

Isolation of RNA and northern blot analysis

Mammary biopsies were taken a week prior to parturition from transgenic founder ewe 001 and again on days 2, 24 and 50 of lactation. Biopsied mammary tissue was immediately placed in liquid nitrogen, and total RNA was isolated by the acid-guanidinium-thiocycanate-phenol-chloroform extraction method, and fractionated on formaldehyde agarose gels (Chomczynski and Saachi, 1987). The RNA was then transferred onto GeneScreen Plus Nylon membranes (NEN Research Products, Boston, MA, USA), hybridized for 12 h at 65 °C with random-primed-labelled cDNA fragments encoding mouse WAP, pig β-casein and pig β-lactoglobulin, washed and exposed to X-ray films.

Loading of total RNA was quantified by hybridizing the blot with an antisense RNA to human 18S ribosomal RNA (Hillis and Dixon, 1991) which cross-hybridizes with the mouse 18S ribosomal RNA and sheep 18S ribosomal RNA. After hybridizing the blot for 4 h in 0.4 M NaCl at 60 °C, followed by washing with 0.2 × SSC (30 mM NaCl, 0.4 mM sodium citrate), and 0.1% sodium dodecyl sulfate (SDS), the filter was scanned with the aid of a Betascope (Betagen Inc., Boston, MA, USA). The WAP hybridization signals were normalized to the 18S signal.

Analysis of mouse WAP

Milk whey proteins were separated under denaturing conditions in SDS 16% polyacrylamide gels and either stained with Coomassie Blue or transferred to nitrocellulose filters. Filters were incubated overnight in TBS (Tris-buffered saline, 20 mM Tris-HCL at pH 7.5, 500 mM NaCl) containing 3% gelatin. The membranes were then probed for 90 min with a 1:200 dilution of rabbit anti-WAP serum, followed by washing and incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS containing 1% BSA for 1 h. The antigen-antibody complexes were stained with nitrobluetetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl at pH 9.5, 5 mM $MgCl_2$ and 100 mM NaCl.

Results and Discussion

Generation of transgenic sheep

Four hundred and forty-three eggs (26, 2-cell) were harvested from donor ewes and a genomic 6.8 kb *Eco* RI DNA fragment containing the mouse WAP gene was injected into pronuclei or nuclei of 281 eggs with visible nuclear structures. All 281 eggs survived injection, based on gross morphological appearance, and 2 to 4 were transferred to the oviducts of each of 118 recipients within 3 h of the time of injection. Thirty-one recipient ewes, into which 80 eggs had been transferred, maintained pregnancy (27%) and 29 lambs and 5 near-term foetuses (2 sets of twins and a single) were produced. The 2 sets of twin foetuses were recovered from recipient ewes that died within a month of their due dates. One ram foetus was transgenic. Three of the 29 live born lambs died within 24 h of birth. One of those lambs was transgenic. Of the remaining 26 healthy lambs (12 ewes, 14 rams) 2 ewe lambs were found to be transgenic. Based on the two surviving transgenic ewes, the overall experimental efficiency was 0.7% (2/281), similar to an efficiency of 0.8% achieved in a contemporaneous experiment (Clements *et al.*, 1994) and previous work (Rexroad *et al.*, 1990).

The founder ewe 001 (carrying approximately three copies of the WAP transgene) and founder ewe 029 (containing approximately seven copies of the WAP) were bred to non-transgenic rams. Ewe 001 produced a

transgenic ram during each of her first two pregnancies and twin full-term foetuses during her third pregnancy. Ewe 029 produced twin ewes lambs during her first pregnancy, one of which was transgenic, and twin full term foetuses during her second pregnancy.

Transcription of the WAP transgene

During lactation, mammary gland biopsies were taken from a non-transgenic ewe, both transgenic ewes, a mouse and a WAP transgenic pig. Total RNA was isolated and was analysed for the presence of WAP mRNA (Fig. 1a). The WAP RNA signal observed in mammary tissue of a mid-lactation (day 10) mouse (Fig. 1a, lane e) and a WAP transgenic pig (Fig. 1a, lane d) were of similar intensity as determined by Betascope analysis of the bands. Steady-state WAP RNA levels of ewes 001 and 029 were 7% and 6% respectively of the WAP RNA signal produced by the mouse band (Fig. 1a, lanes b and c compared to lane e). As expected, no signal was seen in mammary tissue of the non-transgenic ewe (Fig. 1a, lane a). DNA probes for porcine β-lactoglobulin and β-casein RNA cross-hybridized with the corresponding mRNAs in sheep mammary tissue, and were used as loading controls and to verify the integrity of sample RNA. Equal steady-state levels of β-lactoglobulin and β-casein were seen in both ewes. WAP RNA expression in mammary tissue of both ewes was similar to endogenous β-lactoglobulin RNA levels, by Betascope analysis.

Transcription of the endogenous WAP gene in mice is first seen in late pregnancy and is maintained throughout lactation (Pittius *et al.*, 1988). WAP transgenes are frequently not regulated in the same manner as the endogenous gene. Precocious transcription, aberrant hormonal regulation and silencing of the gene during the lactational period have been observed in both transgenic mice and pigs (Burdon *et al.*, 1991b; Shamay *et al.*, 1992). We therefore analysed steady-state levels of WAP RNA in mammary gland biopsies of both transgenic ewes throughout their lactational and dry periods and compared WAP RNA measurements to endogenous milk protein RNAs. Both ewes' profiles were similar. WAP-specific RNA data from ewe 001 is presented in Fig. 1b. Transcripts of WAP and endogenous β-lactoglobulin and β-casein were found one week prior to parturition (Fig. 1b, lane b). WAP RNA reached peak levels by day 24 of lactation (Fig. 1b, lane d) whereas RNA levels for β-lactoglobulin and β-casein had peaked at day 2 of lactation.

Efficient transcriptional activity of the mouse WAP gene in both of the transgenic ewes extends our earlier observation that genetic control elements of this gene are functional in species that do not have a recognizable WAP gene (Shamay *et al.*, 1991; Wall *et al.*, 1991). Clearly, the transcriptional machinery of the sheep alveolar epithelial cells recognizes the WAP gene. The

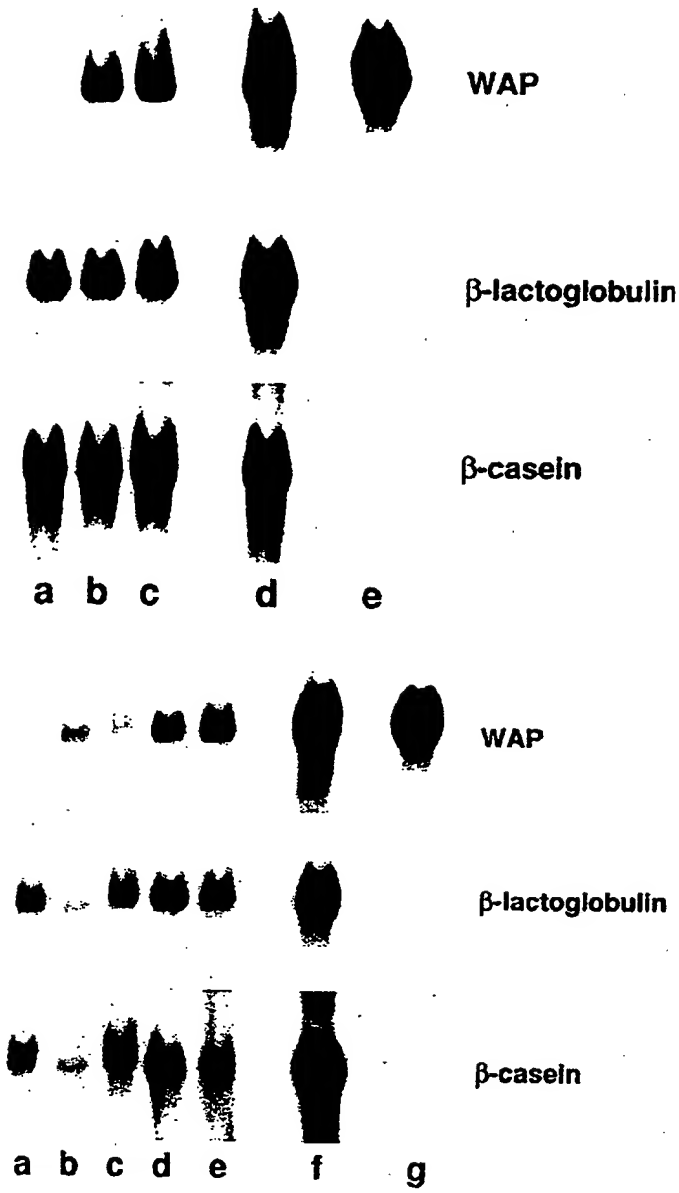

Fig. 1 (a) Expression of mouse WAP RNA in mammary of tissue lactating transgenic sheep. Total RNA was prepared from mammary gland biopsies taken during lactation from a non-transgenic sheep (lane a), ewe 001 (lane b), ewe 029 (lane c), a transgenic pig carrying the mouse WAP gene (lane d, Wall *et al.*, 1991) and from a mouse at day 10 (peak) of lactation (lane e). RNA (10 λg) was separated in a formaldehyde gel, transferred onto a nitrocellulose filter and hybridized with probes for mouse WAP, pig β-lactoglobulin and pig β-casein. (b) Levels of mouse WAP RNA in mammary tissue of a transgenic sheep prior to and during lactation. RNA was prepared from mammary biopsies taken from a lactating non-transgenic ewe (lane a), and from transgenic ewe 001 one week prior to parturition (lane b), at day 2 (lane c), day 24 (lane d) and day 50 (lane e) of lactation. RNA was also prepared from the mammary tissue of a transgenic pig (lane f, Wall *et al.*, 1991) and from a mouse (lane g). RNA (10 λg) was separated in a formaldehyde gel, transferred onto a nitrocellulose filter and subsequently hybridized with probes for mouse WAP, pig β-lactoglobulin and pig β-casein.

two WAP transgenic ewes experienced normal lactational cycles (each ewe produced 4 lambs and transmitted the transgene to their offspring), suggesting that synthesis and secretion of WAP did not interfere with mammary function. In contrast, sows from several lines of transgenic pigs and least two lines of transgenic mice carrying the identical WAP transgene showed aberrant mammary development which resulted in non-functional mammary glands (Burdon *et al.*, 1991b; Shamay *et al.*, 1992). It is not clear whether we have investigated a sufficient number of lines to detect mammary abnormalities, or whether WAP does not interfere with ovine mammary development.

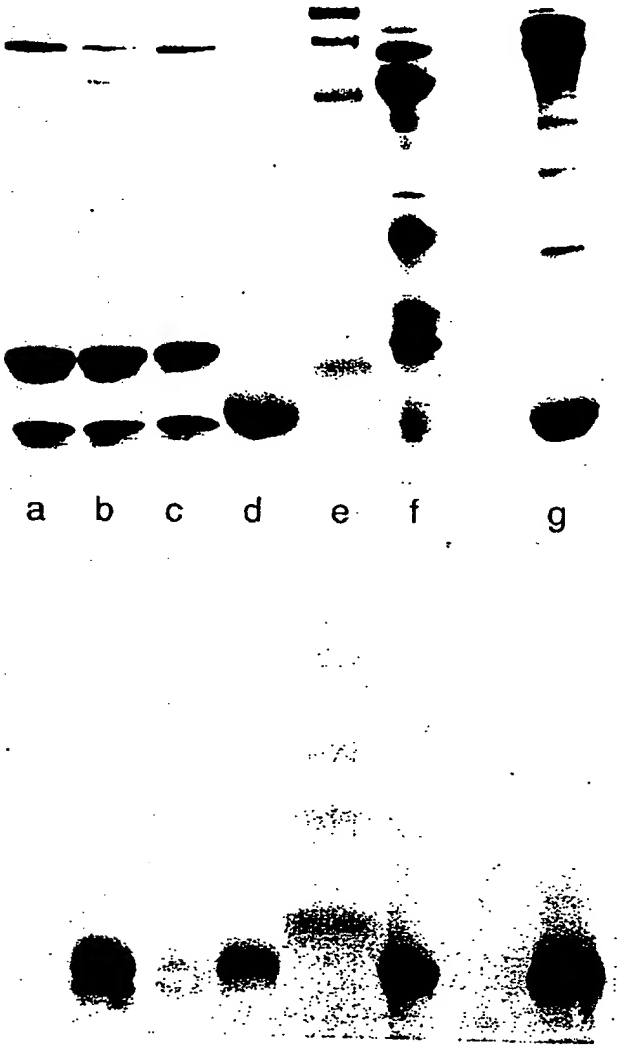

Fig. 2 Secretion of mouse WAP into sheep milk. Milk proteins from a non-transgenic ewe, two transgenic ewes, a transgenic pig, a non-transgenic mouse, and purified mouse WAP were separated in a 16% SDS-polyacrylamide gel, stained with Coomassie Blue (top panel) or transferred electrophoretically onto a nitrocellulose filter and analysed with rabbit anti-mouse WAP antibodies (bottom panel). Lane a, whey fraction from milk of a non-transgenic sheep; lane b, transgenic ewe 001; lane c, transgenic ewe 029; lane d, 5 mg of purified mouse WAP; lane e, prestained molecular weight standards (BRL); lane f, a pig transgenic for mouse WAP; lane g, a non-transgenic mouse.

Synthesis of WAP

Sheep milk whey was analysed for the presence of the mouse WAP on Coomassie-stained SDS-polyacrylamide gels (Fig. 2, upper panel) and with anti-mouse WAP antibodies (Fig. 2, lower panel). The major proteins in the whey fraction of non-transgenic ovine milk are lactalbumin (14 kDa), β-lactoglobulin (18 kDa) and lactoferrin (70 kDa) (Fig. 2, lane a). Since mouse WAP co-migrates with β-lactalbumin, its presence in transgenic ewes was determined immunologically (Fig. 2, lower panel). Whereas no WAP was detected in the whey of a non-transgenic ewe (Fig. 2, lane a), both transgenic ewes secreted mouse WAP into their milk (Fig. 2, lane b and c). The major immunoreactive bands from the sheep samples co-migrated with purified mouse WAP (Fig. 2, lane d), WAP in mouse milk (Fig. 2, lane g) and mouse WAP in the milk of transgenic pigs (Fig. 2, lane f). The lower molecular weight band cross-reacting with anti-WAP antibodies in the milk of transgenic ewes was probably a proteolytic product of WAP which has also been observed in the milk of WAP transgenic pigs (Shamay *et al.*, 1991).

No quantitative assays (e.g. RIA, ELISA) have been developed for measuring WAP concentration. Therefore it was necessary to rely on the more qualitative, western blot analysis, to estimate WAP concentrations in the milk of transgenic sheep. WAP band intensities (excluding the presumptive proteolytic products) of transgenic ewes 001 and 029 (Fig. 2, lanes b and c) relative to the 5 mg of purified mouse WAP band (lane d), were estimated to be 10% and 2% respectively. Therefore the milk samples from ewes 001 and 029 contained approximately 0.5 and 0.1 mg ml^{-1} of WAP, respectively. These estimates of steady-state sheep milk WAP concentrations are in agreement with the WAP RNA levels detected in the tissue biopsies.

Ectopic expression of the WAP transgene

The endogenous WAP gene in mice and WAP transgenes in mice and pigs are tissue specific, expressed predominantly in mammary tissue and to a lesser extent in salivary glands (< 1% of mammary gland levels). To evaluate the tissue expression pattern of the WAP transgene in sheep, RNA was prepared from ten tissues for northern blot analysis. Tissues were recovered from founder ewe 029 following an unsuccessful attempt to rescue her twin lambs by caesarean section within a week of her expected parturition date. WAP gene expression was detected in a variety of tissues. The RNA samples were too degraded to quantitatively compare WAP expression in the various tissues with that of its expression in mammary gland tissue. Qualitatively, it appeared that

ectopic expression was highest in spleen and salivary gland, intermediate in liver, lung, kidney and heart muscle, lowest in bone marrow and not detectable in skeletal muscle or intestine.

This unexpected finding is based on tissues harvested under less than ideal conditions (the ewe had died) from one animal and therefore must be interpreted with caution. Tissues were also recovered from the other founder (001) which also died in the night following a caesarean section. However, the extracted RNA was degraded and could not be analysed. Though the poor quality of the RNA from ewe 029 precluded quantitative analysis, there is no question that WAP was expressed in tissues other than the mammary gland. It is possible that the ectopic expression observed was an artifact caused by the so-called transgene position effect. However, it is equally likely that sheep possess regulatory factors, in a variety of tissues that interact with the WAP gene. Additional lines of transgenic sheep will have to be analysed to distinguish between the two possibilities.

Health problems

There was an unusually high morbidity rate in this study. Founder 001 died near the end of her third gestation and ewe 029 died at the end of her second gestation. Dead full-term twin foetuses were recovered from both ewes the day before they died. No specific pathology was detected at the time of necropsy that could account for their deaths. It is not unreasonable to assume that the stress of carrying dead twin foetuses and the subsequent surgical trauma contributed to the founders' deaths. However, in a previous study, one of six founder pigs, carrying the same transgene also died at parturition (Shamay *et al.*, 1992).

As a result of her two successful pregnancies, ewe 001 produced 2 offspring, both of which were transgenic rams. One of the rams died at 7 months and the other 12 months of age. Necropsies revealed no gross organ abnormalities. Ewe 029 produced twin ewes, one of which is transgenic. That ewe, 211, is now two years old and appears to be in good health, and was recently bred.

Ectopic expression of the transgene was observed in tissues from founder ewe 029. Therefore, it is possible that expression of WAP in tissues other than the mammary gland contributed to the deaths of these transgenic animals. A recent study, in which we purposely expressed WAP in tissues other than the mammary gland, provides some support for that hypothesis (Hennighausen *et al.*, 1994). No increased morbidity or overt health problems were observed, but we detected hyperplasia and dysplasia of coagulation gland epithelium, one of the tissues that expressed a moderate amount of WAP RNA. However, because the function of WAP is unknown, it is difficult to speculate on a mechanism that might explain these observations.

In mouse and pig studies where the same transgene was used, we have observed a correlation between premature mammary gland expression of the WAP gene and the milchlos phenotype, a syndrome characterized by incomplete development of secretory epithelium (Burdon *et al.*, 1991b and Shamay *et al.*, 1992). We have inferred from these observations that WAP may play a role in mammary gland development. If that is the case, ectopic expression of the WAP gene may have deleterious developmental consequences in other organs.

Crossing species boundaries

Transgenic mice have been employed extensively to study regulatory features of genes from a variety of species, including humans, rats, cattle, pigs, sheep and goats. However, there have been only a few studies in which the regulation of a transgene has been compared in several species. Using WAP transgenes allows us to address fundamental issues of transgenesis and gene regulation across species boundaries. Surprisingly, expression of a mouse WAP transgene was more efficient in pigs (Shamay *et al.*, 1991; Wall *et al.*, 1991) than in mice (Burdon *et al.*, 1991a). Whereas only one half of the transgenic mouse lines expresses the WAP transgene, all six lines of pigs and both lines of sheep express the transgene. Mammary specificity was maintained in mice and pigs, suggesting that the molecular basis of mammary-specific gene expression has been conserved. However, the data presented here suggest that sheep and possibly other ruminants (cattle and goats) contain regulatory factor(s) that interact with the WAP gene in a variety of tissues.

The data presented here and in other work (Burdon *et al.*, 1991a; Shamay *et al.*, 1991; Jhappan *et al.*, 1993; Velander *et al.*, 1993) highlight some of the problems associated with the murine transgenic system as a predictive model. For the mouse, WAP transgenes behave somewhat erratically (precocious expression and lower than endogenous levels), but neither in mice nor pigs was tissue specificity lost. The one ewe from which RNA from a variety of tissues could be analysed, clearly demonstrated ectopic expression of the WAP transgene. Since only one animal was analysed, it is not clear whether the WAP gene generally loses its mammary-specific expression in the background of transgenic sheep, or whether the WAP transgene integrated within a chromosomal region that fosters widespread expression. Unfortunately, due to the high cost of producing transgenic sheep, it is unlikely that this question will be resolved until the efficiency of generating transgenic sheep is substantially improved.

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Appendix G

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(54) Title: METHOD FOR DEVELOPMENT OF TRANSGENIC GOATS

(57) Abstract

The invention features a method which includes the following steps: (a) introducing a transgene into a zygote of a dwarf goat, (b) transplanting the zygote into a pseudopregnant non-dwarf goat, and (c) allowing the zygote to develop to term. In another aspect, the invention features a method which includes the following steps: (a) introducing a transgene into an embryo of a dwarfgoat, (b) transplanting the embryo into a pseudopregnant non-dwarf goat, and (c) allowing the embryo to develop to term.

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METHOD FOR DEVELOPMENT OF TRANSGENIC GOATS

Field of the Invention

The field of the invention is methods for development of transgenic goats.

Background of the Invention

Selective breeding of domesticated animals, based on phenotypic traits, has led to significant genetic improvement in a variety of breeds. For example, selective breeding techniques have resulted the production of diary cows which produce more milk and produce milk having improved fat and protein profiles. Reproductive technologies including artificial insemination and embryo transfer (i.e., transferring an embryo to an animal that did not produce it) have contributed to this genetic improvement. Advanced reproductive and molecular technologies, e.g., embryo cloning, and marker assisted selection (genetic screening), allow even greater control over the selection of desirable traits. However, these practices only allow selection and propagation of traits which are already in the gene pool of the species. In order to replace or modify existing genes or insert genes for traits which are not present in the gene pool of the species, a transgene must be introduced into the animal. Transgenes can be used to introduce disease resistance, alter the composition of animal derived products (milk, serum, etc.), produce pharmaceutical or nutriceuticals, or for other purposes.

A number of techniques can be used to create transgenic animals. Among the techniques which have been successfully used are: pronuclear injection of the transgene, nuclear transplantation, and injection of genetically altered stem cells into host embryos (chimera production).

Pronuclear injection is a commonly used procedure for germ line insertion of genes. While this technique is attractive because it can be used successfully with a range of animals, the inability to control integration of the transgene and the large number of ova which must be injected to obtain even a single transgenic offspring combine to make the technique rather inefficient, at least for animals other than mice.

Moreover, animals which are deemed transgenic, e.g., by Southern analysis, may be mosaic, may contain the transgene but not express it, may express it in an undesirable manner, or may express correctly but fail to transmit it to offspring. Despite these difficulties, transgenic mice, sheep, goats, cattle and pigs have been produced using pronuclear injection.

Transgenic mice have been created by genetically manipulating murine embryonic stem cells (ESC), e.g., by injecting a transgene into the ESC, and then injecting the altered embryonic stem cells into a host embryo. The resulting mice are mosaics in which genetically altered cells contribute to a greater or lesser extent to the somatic and germ cells.

Nuclear transfer is a third approach to the generation of transgenic animals. In this technique the nucleus of a donor cell is introduced into a recipient oocyte. Offspring have also been reported in the bovine and sheep from cultured inner cell mass (ICM) cells and embryonic disks, respectively, using the technique of nuclear transfer.

Embryo transfer is a technique in which an embryo taken from a donor animal is transferred to a recipient animal who brings the embryo to term. Embryo transfer has successfully produced offspring when embryos from dwarf goats were transferred into standard goats Sugie et al., 1970.

Summary of the Invention

The invention features a method which includes the following steps: (a) introducing a transgene into a zygote of a dwarf goat, (b) transplanting the zygote into a pseudopregnant non-dwarf goat, and (c) allowing the zygote to develop to term. In another aspect the invention features a method which includes the following steps: (a) introducing a transgene into an embryo of a dwarf goat, (b) transplanting the embryo into a pseudopregnant non-dwarf goat, and (c) allowing the embryo to develop to term.

In another aspect, the invention features a method which includes the following steps (a) introducing a transgene into an zygote of a dwarf goat, (b)

transplanting the zygote into a pseudopregnant dwarf goat, and (c) allowing the zygote to develop to term. In another aspect, the invention features a method which includes the following steps: (a) introducing a transgene into an embryo of a dwarf goat, (b) transplanting the embryo into a pseudopregnant dwarf goat, and (c) allowing the embryo to develop to term.

In preferred embodiments the method also includes: (d) breeding the offspring to produce a transgenic dwarf goat. In other preferred embodiments the introducing of the transgene into the embryo is by introducing an embryonic stem cell containing the transgene into the embryo; the introducing of the transgene into the embryo is by infecting the embryo with a retrovirus containing the transgene; the introducing of the transgene into the zygote is by injecting the pronucleus of the zygote with the transgene.

In other preferred embodiments at least four zygotes are transplanted into the pseudopregnant non-dwarf goat and at least four embryos are transplanted into the pseudopregnant non-dwarf goat.

In another aspect the invention features dwarf goat embryonic stem cells.

By "transgene" is meant a DNA sequence introduced into the germline of non-human animal by way of human intervention such as by any of the methods described herein.

By "dwarf goat" is meant a Nigerian Dwarf goat or a Pygmy goat or any other goat of small size comparable to that of a Nigerian Dwarf goat or a Pygmy goat. Suitable goat breeds preferably weigh approximately 80 lbs or less at maturity and weigh 2.0 kg, more preferably 1.7, or 1.5 kg at birth. Suitable breeds are of a fetal size and neonatal size which permit a non-dwarf goat (i.e., a standard goat) to which the dwarf goat embryo or zygote are transferred to bear 3 or 4, more preferably 5 or 6, dwarf goats in a single pregnancy. Achondroplastic dwarf goats are suitable for use in the method of the invention as are dwarf goats whose small stature is due to some other cause.

Because several dwarf goat embryos can be implanted in a single pseudo-

pregnant standard goat, the method of the invention provides a means by which to decrease the number of recipient animals required for the production of transgenic goats. Transfer of multiple dwarf embryos to standard goats as recipients results in an increase (e.g., 2- to 4-fold) in the number of offspring per recipient, compared to the implantation of standard goat embryos into standard goat recipients. This represents a significant increase in production efficiency and a significant decrease in cost of recipient animals compared to other systems, such as the transfer of standard goat embryos to standard goat recipients. Moreover, dwarf goats have characteristics, including lack of seasonality, early onset of sexual maturity, and small fetal and neonatal size, and good milk yield, which are highly desirable in transgenic animals used for the production of pharmaceuticals or nutriceuticals in milk. Lack of seasonality and early onset of sexual maturity decrease the generation interval as compared to other dairy ruminants. Using the method of the invention, a heterologous gene product can be expressed in the milk of a transgenic animal within a time-frame which is the shortest of any dairy ruminant (e.g., a year before that of transgenic dairy cattle).

It is the small fetal and neonatal size of dwarf goats which allows for the implantation of several embryos into a single standard goat recipient. Dwarf goats are a desirable system for the production of transgenic goats even when dwarf, rather than standard, goats are used as the recipient for embryos or zygotes. This is because a dwarf goats naturally produce more offspring in single pregnancy than do standard goats.

The method of the invention can be used with any method for producing transgenic dwarf goats, including: pronuclear injection (or any other means for introduction of a gene into a cell), nuclear replacement (embryo cloning), and injection of genetically-manipulated embryonic stem cells into embryos (chimera production). The method of the invention can also be used to develop other advanced reproductive and genetic technologies such as homologous recombination. The

method of the invention has numerous advantages over other ruminant-based transgenic systems.

The method of the invention preferably entails expansion and propagation of dwarf goat progeny, transgenic (or otherwise genetically altered such that at some of the cells harbor a gene that does not naturally occur in the species) transfer of embryos or ova or zygotes to standard goat does. In general, 4 to 6 or more dwarf goat embryos can be transferred to a standard recipient doe using the method of the invention. This represents an significant improvement relative to the use of standard goats where only 1 or 2 standard goat offspring are born to a standard goat recipient.

Another aspect of the invention entails transfer of genetically manipulated dwarf goat embryos to dwarf goat does.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

A. Dwarf Goats

Dwarf goats are indigenous to India, Arabia, China, West Indies and Africa (especially Nigeria, Ghana, and the Cameroons). Dwarf goats predominate in western Africa due to their natural resistance to the tse tse fly which destroys other goat types. This West African goat is also called the Fouta Djallon, Cameroon, Nigerian or tree goat.

Breeders in North America selecting for slightly different show traits have characterized two show breeds of Dwarf goats, the Pygmy goat and the Nigerian Dwarf goat. Nigerian Dwarves have slightly longer necks and legs and their barrels are not as wide or deep as those of Pygmies. Breeders of Pygmy goats also select for a more limited range of coat color. These show breeds are both derived from western African dwarf goats and have similar reproductive and milk production traits (Tables 2 and 3). Dwarf goats useful in the method of the invention include Nigerian Dwarf Goats, Pygmy Dwarf Goats, and other dwarf goats which are of a size which permit a standard goat to carry 3 or more to term in one gestation.

The American Nigerian Dwarf Goat Organization (Alvarado, TX; Internet Address: rivoire@abn.unt.edu) provides information concerning Nigerian Dwarf goats including breeder lists. According to the American Nigerian Dwarf Goat Organization, ideal does are 17" to 19" high (21" maximum), and ideal bucks are 19" to 20" high (23" maximum). Their ideal weight at maturity is 75 lbs. Of course, dwarf goats useful in the method the invention need not meet these stringent criteria. Nigerian Dwarf Goats average 2 lbs at birth. Bucks can reach sexual maturity at 7 weeks. Does can typically be breed at 7 - 8 months, although it is possible to breed them earlier. Nigerian Dwarf Goats are registerable with the American Goat Society, the International Diary Goat Registry, and the Canadian Goat Society. Additional Nigerian Dwarf Goat information is available on the Internet at http://www.okstate.edu/.../nigndwrf.html.

The National Pygmy Goat Association has set standard for the breed which have been accepted by the American Goat Society. According to these standards an adult buck is 16.0" to 23.6 high at the wither, and an adult doe is 16.0" to 22.4" high at the wither.

Information concerning both the Pygmy Goat and the Nigerian Dwarf Goat can be found in "The Pygmy Goat in America with the Nigerian Dwarf." (Alice Hall, Hall Press, San Bernardino, CA, 1992; ISBN: 0-932218-13-X; Library of Congress Catalog Card Number: 82-90126).

The dwarf goats are homozygous for small size. Dwarfism appears to be controlled by three genes (Blanks, *A Morphological, Physiological and Genetic Investigation of the African Pygmy Goat*, Doctoral Thesis, Oregon State University, 1971). At birth, the typical dwarf kid is less than half the size of a standard kid at birth. Dwarf goat birth weights average 1.4 kg for females and 1.6 kg for males while for standard Saanens the birth weight are 3.6 kg for females and 3.8 kg for males. Hybrid dwarf-standard kids are intermediate between parental phenotypes.

Table 2. Comparison between Dwarf and Standard breed Goats

Dwarf Goat (e.g., Pygmy, Nigerian)	Standard Goat (e.g., Saanen, Alpine)
	Differences
Breeds all year	Most are seasonal breeder (anestrous 4 months)
mature weight 20-35 kg (female)	mature weight 75 kg
sexually mature 3-6 months	sexually mature 10-12 months
	Similarities
gestation 5 months	gestation 5 months
milk production high (1000 lbs/yr)	milk production high (2700 lbs/yr)
twinning, triplets typical	Singles, twinning typical

Shorter Generation Times

Dwarf goats have a shorter generation time than other dairy ruminants (standard goats, sheep, cows) owing to their sexually precociousness. Dwarf goats of both sexes are mature rapidly. The females generally begin to cycle at two to six months of age, compared to nine months for standard goats, and are capable of carrying a pregnancy to term if impregnated at this time. Similarly, male Dwarf goats produce viable semen by two to three, compared to five months for standard goats. Standard management practice with Dwarf goats mandates that the males be removed from their litter mates by two months of age to prevent young bucks from mating with their sisters prior to weaning.

Early puberty is important because it allows a transgenic female to be impregnated at three to four months after birth, allowing for early milk production at kidding. The entire process beginning at microinjected ovum to lactating transgenic female is 14 months (five months for fetus gestation, four months for doe growth, and five months for doe gestation). Moreover, lactation can be induced hormonally, thus even further shortening the time required to obtain transgenic milk (five months for fetus gestation and two to four months for doe growth).

Lack of Seasonality

The Dwarf goat is sexually active throughout the year, a fact that further contributes to their shorter generation intervals. Standard goats in temperate climates have defined periods of sexual activity. In the northern hemisphere, standard goats have a defined rutting season from September to March with "heats" occurring some 18-21 days apart. In contrast, Dwarf goats are polyestrous (i.e., they do not show a period of sexual inactivity), probably because they evolved in the tropics where the seasonal effects on reproduction are slight. This lack of seasonality is an important aspect of Dwarf goat physiology because pubertal animals can be propagated year round either through natural breeding, superovulation, embryo transfer, or through frozen embryo storage with subsequent transfer. This is particularly advantageous for increasing numbers of transgenic animals. Some standard breeds of goats are less seasonally dependent than others and can be used as recipients throughout most of the year.

Lactational Performance

The lactational performance of transgenic Dwarf goats is sufficient to allow them to be a viable model for heterologous protein production. Typical lactation produces on average 1.0-1.5 L/day for 10 months, a remarkable amount considering the Dwarf goats' small body size. This lactation performance is thus sufficient to satisfy the requirement for gram quantities of protein. The American Goat Association has set production records for Pygmy goats as producing 1/3 of the larger dairy goat breeds. The International Dairy Goat Registry has set similar production standards for Pygmy and Nigerian dwarf goats. Although production standards are from 460 to 550 lbs (209 to 250 kg) for yearlings, older does may produce more. Does producing over 1,000 lbs (454 kg) with high butterfat (5.5%) and protein (4.2%) are not uncommon (*Dairy Goat Journal*, 1993 and Nigerian Dwarf Goat Association). Recent evaluation of the milk of a Nigerian dwarf doe from our herd found average of 5.6% fat, 4.3% protein and 4.9% lactose (8 samples). This surpasses the standard goat average protein content of 3.0% for a Quebec dairy reporting lactational yields for 34 milking dairy goats (*Canadian Goat Society Quarterly*, Nov. 1994).

Table 3. Production standards for yearling dwarf goats

Association	Type	lbs per lactation (305 days)
American Goat Association	pygmy	500
International Dairy Goat Registry	pygmy	460
	Nigerian dwarf	550

(Hall, The Pygmy Goat in America, *supra*)

Small Physical Size

In addition to the advantages in production, the small physical size of Dwarf goats means that less space required to house lactating Dwarf does. In addition, their small size and personable nature also make handling the animals easier.

B. Multiple Birth Goat System

The multiple birth goat system is based on the use of dwarf goats. Because dwarf goat fetuses and neonates are small, a standard recipient doe can gestate and give birth to multiple Dwarf offspring successfully. Even dwarf does can successfully carry 3 to 4 dwarf goat offspring. Either approach results in increased multiplicity of offspring and shorter generation intervals compared to producing standard goats in standard goats. The method involves the collection of Dwarf goat embryos and their transfer into synchronized standard recipient goat does or into synchronized dwarf goat recipients. In one study we were able to recover a total of 67 dwarf goat embryos from six dwarf goat donors (average number embryos from each donor = 11.2). In another study a total of 16 dwarf goat embryos were transferred to three standard goat recipients (average number embryos to each recipient = 5.3), two of whom maintained pregnancy.

Several steps are involved in the method of the invention. These steps include: synchronization and superovulation, embryo recovery, and embryo transfer. Not all of these steps are necessarily involved in every implementation of the multiple birth goat system. The precise steps and techniques involved depend on use to which

the system is being put. In many cases the dwarf goat embryos are manipulated (cloned, microinjected, etc.) prior to transfer to the recipient doe (which may be a standard or dwarf goat).

Synchronization and Superovulation

The goats (standards and Dwarf) can be synchronized and superovulated by any established regime known by those skilled in the art. The following hormonal regime is one example of the methods which can be used. Sponges containing 60 mg of methoxyprogesterone acetate were inserted into the vagina of standard and dwarf goats and left there for 17 days. For three days, starting on day 15 of the sponge treatment, the dwarf goats (embryo donors) received two injections of follicle stimulating hormone (FSH) a day in a decreasing dosage (5 mg, 4 mg, 3 mg, 2 mg, 2 mg, and 2 mg, with 200 I.U. PMSG on day 2). Breeding was achieved by having the buck with the does on day 18 and 19. Alternately the doe can be inseminated artificially. Standard goat recipients were not breed, but were synchronized using the sponges. Lower doses of FSH or pregnant mare serum gonadotropin (PMSG; 400 I.U.) can also be used in the synchronization of the recipients to ensure ovarian response. The embryos are collected and transferred five days following breeding.

Recovery of Embryos

Embryos were recovered and transferred surgically using standard procedures. The goats were fasted 24 hours prior to surgery then given 0.66 mg atropine sulfate. Anaesthesia was induced with intravenous administration of diazepam (1 mg/kg body weight) and ketamine (5 mg/kg). Anaesthesia was maintained with halothane via endotrachial intubation. For morula and blastocyst stage embryos (day 4-6 post insemination) the uterus was exposed and the proximal uterine horn occluded with a Foley catheter (Fr. 10 or 12 for standard does and Fr. 10 for Dwarf does). A Fr. 3½ Tom Cat catheter was inserted at the utero-tubal junction and 10 to 20 ml of media was introduced into the uterus. This fluid was drained by gravity into a sterile receptacle. Both horns of the uterus were treated in the same way. Earlier staged embryos can be recovered in a similar manner by flushing the oviducts (day 1-3 post

insemination). Other embryo recovery methods can also be used in the method of the invention.

Embryo Transfer

The dwarf goat embryos were introduced into synchronized recipient standard goats either by a surgical procedure similar to embryo recovery, or by laparoscopic or nonsurgical procedures (Pendleton et al., *Louisiana Agriculture*, 29:6-7, 1986, describes such procedures). In the surgical procedure the exposed uterus was pierced with a needle (18 g) and a Tom Cat catheter carrying the embryos in a minimal amount of media was inserted through the puncture hole and carefully threaded into the uterine horn. The embryos were expelled into the uterine horn and the catheter carefully removed.

Various embryo transfer techniques applicable to diary goats are described by Youngs et al. (Youngs et al., Embryo Transfer in Dairy Goats, in *The Proceedings of the Intl. Goat Production Symposium*, Florida A & M Univ., Tallahassee, FL, 1990). This reference also describes related techniques including: embryo cyropreservation, embryo splitting, *in vitro* embryo culture, gene transfer, nuclear transplantation, *in vitro* fertilization, and embryo sexing. Various embryo transfer techniques applicable to diary goats are described by Youngs et al. (*supra*). Braun et al. describes useful methods for non-surgical collection of goat embryos. (Braun et al., Non-surgical Embryo Collection in the Dairy Goat, in *The Proceedings of the Intl. Goat Production Symposium*, Florida A & M Univ., Tallahassee, FL, 1990).

C. Embryo Manipulation

The embryos recovered from the Dwarf embryo donor may be manipulated prior to embryo transfer using the following methods or other methods known to those skilled in the art. In general, any method for the production of transgenic goats can be used in the method of the invention.

Microinjection

Microinjection of the transgene into the pronuclei of zygotes (produced either *in vivo* or *in vitro*) is a preferred method for the generation of transgenic animals.

The pronuclei can be visualized by differential interference contrast which can be aided by briefly centrifuging the zygotes. DNA solution, for example an expression vector with the casein control element driving expression of the desired transgene, is injected into one of the pronuclei. Injected zygotes can either be transferred surgically to the oviducts of recipients or cultured *in vitro* until the morula or blastocyst stage for surgical or non-surgical transfer, possibly following embryo biopsy. *Guide to techniques in Mouse Development* (*supra*) provides detailed descriptions of a wide variety methods which can be used to produce transgenic animals and manipulate embryos. Simons et al. describes examples of the use of microinjection of sheep pronuclei and the production of transgenic sheep which produce human clotting factor IX and α1-antitrypsin (*Bio/technology*, 6:179, 1989). Meade et al. (U.S. Patent No. 4,873,316) describes a microinjection techniques and expression systems suitable for expression of recombinant proteins in mammal's milk .

Ebert et al. (*Bio/technology* 12:699, 1994) describe the induction milk containing of human tissue plasminogen activator from the mammary gland of transgenic goats from a first generation transgenic male. Ebert et al. (*supra*) also describe a useful method for induction of lactation in males using a hormonal regime. This method permits early assessment of a transgene expressed in the mammary glands, even in males.

Embryo biopsy

Under some circumstances it is desirable to obtain a few cells from an embryo. The cells can be used to determine the gender of the embryo, determine whether the transgene is present, and assess the structure of the transgene (e.g., whether it has been rearranged). One to a few cells can be removed, preferably at the 16 cell or greater stage, using micro instruments. Genotyped embryos can be transferred to recipients or can be stored frozen for later transfer.

In vitro development of oocytes

Keskintepe et al. describe a method for developing morulae *in vitro* from immature goat oocytes (*Zygote* 2:97, 1994). This method can be used to provide

zygotes and embryos for gene manipulation, host embryos for chimera production, unfertilized recipient oocytes for use in nuclear transfer, and embryos and oocytes for use in other techniques.

Expansion of transgenic embryos by chimera production

Transgenic dwarf goat embryos, including those produced by pronuclear injection, nuclear transfer, or obtained from a transgenic animal, can be split into small sets of cells, preferably 3 to 6 cells, but also as few as one and as many as half of the embryo's cells. Each small set of cells (or each cell) can be injected into an intact host goat embryo or sandwiched between the separated cells of a host goat embryo for the production of chimeric goats. The host embryo can be produced *in vivo* or *in vitro*. Dwarf goat embryos are preferred hosts, although standard goat embryos can also be used, particularly where the host cells are directed to placental formation. It can be diploid or tetraploid. By controlling the ratio of injected embryo to host embryo cells, stage of host (younger), ploidy of host (tetraploid) or differentiation of host (trophectoderm) and embryo (inner cell mass), the host embryo can be directed towards placental formation while the transgenic (injected) embryo can be directed towards fetal contribution.

Embryonic stem cell isolation and culture

Embryonic stems cells (ESC) are very useful in the production of transgenic animals. They can be genetically transformed and then used to form chimeric embryos by blastocyst injection, morula injection, morula aggregation, or other techniques (*see Guide to Techniques in Mouse Development, supra*). When ESC harboring the transgene are incorporated into the germ line and participate in the production of reproductive cells, the offspring produced by the chimeric animals will be transgenic. ESC are have several advantages: 1) they permit increased efficiency of transgenic animal production; 2) they can be transformed *in vitro*; 3) they can be screened for the presence of the transgene (Robertson, *Biology of Reproduction* 44:238, 1991); and 4) they can be propagated so that one can generate many identical transgenic animals. The use of ESC makes it possible to replace an existing gene with

a genetically altered gene by homologous recombination (Thomas et al., *Cell* 51:501, 1987). Pieper et al. (*Nucleic Acids Res.* 20:1259, 1992) describes methods for introducing a transgene into a murine zygote by homologous recombination.

The following references provide information concerning the production of ESC and their use in the production of transgenic animals: Wheeler et al. (*Reprod. Fertil. Dev.* 6:563, 1994); Reed et al. (PCT Application No. PCT/US93/08878); Wheeler (PCT Application No. PCT/US94/05529); Iannoccone (PCT Application No. PCT/US94/09787); and Evans et al (PCT Application No. PCT/GB89/01103).

Dwarf goat ESC can be produced as follows. Dwarf goat embryos, preferably at the blastocyst or younger stages, are cultured after removal of the zona pellucida, either through natural hatching process or mechanical removal, on an appropriate feeder cell line such as embryonic fibroblast feeder layers (mouse, STO, goat, etc.). Embryos attach to the monolayer and proliferate as an undifferentiated cell line (embryonic stem cells). These cells can be propagated as a cell line, stored frozen, or transfected with DNA construct by any of known DNA transfer techniques (i.e., these cells are amenable to all somatic cell manipulations known to one trained in the art). ESC can be derived from morulae (Eistetter, *Dev. Growth and Diff.* 31:276, 1989). ESC can also be isolated from primordial germ cells (Matsui et al., *Cell* 70:841, 1992).

Putative dwarf goat ESC were derived as follows. Morula-staged and/or blastocyst-staged embryos were collected from superovulated dwarf goat does on day 6 (esterus = day 0). The embryos were placed into Embryonic Stem Cell Medium (ESCM; MEM (GIBCO) supplemented with nonessential amino acids, 0.1mM β-mercaptoethanol, and 15% bovine fetal calf serum (FCS) over a feeder cell layer. In this example the feeder cell layer was mitomicin blocked STO cells (available from the American Type Culture Collection, Rockville, MD). Alternately, mouse embryonic fibroblastic cells, or goat embryonic fibroblastic cells could be used as a feeder layer.

Over a period of 7 days the embryos hatched from their zonae pellucidae and

attached to the feeder layer. After a few days (up to and over 2 weeks) attached cells were removed mechanically by cutting into pieces with sharp edge, glass or metal needle or blade, and lifting off the surface. These pieces were then enzymatically dispersed (using trypsin or protease) into small clumps of cells and replated onto plates of fresh feeder cells. The enzymatic treatment can be omitted and the small pieces passaged directly to the new feeder layer.

After 7 to 14 days the embryonic stem cells colonies were passaged again as described above. This passaging step is repeated as needed. Colonies can be passage while small and undifferentiated or can be allowed to almost reach confluency. Large colonies may have areas of differentiated and undifferentiated cells. Undifferentiated cells can be preferentially removed for continued passage.

ESC can be frozen in 10% glycerol and 90% ESCM. ESC lines can be restarted from frozen cells. Frozen cells are thawed rapidly, washed free of cryoprotectant and plated onto fresh feeder layers.

As an alternative culture method, established ESC (after 1 passage) can be plated onto gelatin coated tissue culture plate instead of feeder cell layers. The ESC medium is supplemented with BRL cell conditioned medium (60% BRL conditioned MEM supplemented with 40% ESCM and 0.1 mM β-mercaptoethanol).

The ESC prepared as described above have a large nuclear to cytoplasmic ratio. At high cell numbers, the ESC grow in a flat monolayer with indistinct cellular edges. Colony edge is distinct and smooth. Cell size is less than or equal to 21 μm. When plated as single cells or in small clumps they form a small mound which will later expand to large flat colony as numbers increase. Undifferentiated cells are alkaline phosphate positive and form simple embryoid bodies spontaneously as cell numbers (colony size) increases. Some colonies may spontaneously differentiated into large flat (trophectoderm-like) cells and/or with cells which have morphological characteristics of nerve cells and/or muscle cells.

Embryonic stem cell-chimera production

Embryonic stem cell, preferably selected for the appropriate incorporation of a

transgene, are injected into a host embryo, preferably when the host embryo is at the morula or blastocyst stage, although injection can occur when the embryo is even younger. The ESC used are preferably from selected colonies which are separated into small clumps of cells (preferably five to fifteen cells) either by mechanical or enzymatic (pronase or trypsin) treatment. These cells would be injected into the blastocoel of blastocyst staged embryos or under the zona into the mass of morula or younger staged host embryos. Alternately, zona free morula (or younger) embryos can be cultured with ESC separated by enzymatic treatment, allowing ESC to be incorporated into the embryo. Host embryos can be *in vivo* or *in vitro* produced, diploid or tetraploid. Guide to Techniques in Mouse Development (*supra*) describes techniques employing ESC in the production of transgenic animals.

Nuclear transfer for multiplication of embryos

Nuclear transfer is an alternative method for propagating transgenic animals. The nuclear donor source can be either a cell taken from a transgenic embryo (following pronuclear injection or derived from a transgenic dam/sire) or a transgenic embryonic stem cell. The cytoplast/host source can be any goat oocyte, *in vitro* or *in vivo* matured.

The host oocyte is enucleated (metaphase II chromosomes removed) either by microsurgical or by centrifugation methods. The resulting host cytoplast is activated by any number of means (cold shock, electrical pulse, calcium ionophore - DMAP treatment, ethanol, etc.) prior or post nuclear transfer depending on the cell cycle stage of the donor nucleus. Generally, embryonic cells are transferred to a preactivated cytoplast while the ES cell-cytoplast are activated post transfer and fusion. The donor nuclei are obtained by either mechanical or enzymatic (for example, trypsin, protease) separation of the donor embryo or cell line. The individual cells (karyoplasts) are transferred to the enucleated oocyte (cytoplast) under the surrounding zona pellucida such that there is contact between the plasma membranes of the karyoplast and cytoplast. The karyoplast and cytoplast are fused by any of several methods including but not limited to, electrofusion, PEG, fusogenic proteins or viruses, etc. The new

zygote is cultured to an appropriate stage for transfer to a recipient animal or frozen storage. An alternate method to karyoplast/cytoplast fusion is that the donor nucleus can be injected directly into the ooplasm of the enucleated oocyte (Collas et al., *Molecular Reproduction and Development* 38:264, 1994). The new zygotes produced by these nuclear transfer techniques can also be combined with a host embryo (in the manner described above) to produce chimeras. Prather et al. (U.S. Patent No. 4,994,384) and Massey (U.S. Patent No. 5,057,420) describe nuclear transfer methods. Tatham et al. (*Biology of Reproduction* 53:1088, 1995) describes additional nuclear transplantation methods.

D. Use of Transgenic Goats

The transgenic dwarf goats produced by the method of the invention can be used to produce useful human therapeutic proteins (e.g., human growth hormone) and veterinary therapeutic proteins (e.g., IL-6) in the milk of the dwarf goats. Production of the heterologous protein in a mammal facilitates post-translational modification of the protein and obviates expensive cell culture media used in *in vitro* methods of protein production. The invention also offers the advantage that the heterologous protein can be produced in large quantities. Transgenic goats can also be used to alter the characteristics of milk.

Transgenic goats can be used for many of the same purposes for which other transgenic animals have been used. The following references describe a variety of uses for transgenic animals: Sarvetnick et al. (PCT Application No. PCT/US94/04708); Bjursell et al. (PCT Application No. PCT/SE93/00515); Lonberg (PCT Application No. PCT/US94/04580); and Abraham et al. (PCT Application No. PCT/GB94/00569).

For example, expression of an appropriate transgene can cause alterations in the protein, lipid, or carbohydrate content of the milk. Useful milk products, such as those having a reduced lactose content, can readily be produced. In addition, where the transgene expresses β-galactosidase derived from *Aspergillus niger*, the enzyme is particularly useful for hydrolyzing lactose at an acidic pH (at pH 3-4). Accordingly, a

sample of milk including this enzyme is particularly useful for reducing the lactose content of a second sample of milk by simply mixing the two milk samples together.

Where the heterologous enzyme is an aspartic protease, the milk is particularly useful for producing cheese. Such proteases decrease the time required for milk to be clotted by rennet. Aspartic proteases can also increase the yield of cheese. The expression of a bovine β-casein in milk can also improve cheese yields. In addition, the production of bovine β-casein or other heterologous proteins (e.g., lactoferrin or lysozyme) in milk can increase the nutritional value of the milk.

Because heterologous enzymes can be tethered to the mammary epithelial cell membrane, the invention also enables the production of modified milk, while decreasing the concern that such milk contains heterologous enzymes. Tethering enzymes to the mammary epithelial cell membrane also decreases the demand placed on the cell's synthetic machinery, since the enzyme can act on components of the milk without being secreted into the milk.

E. Transgenes

Useful promoters for the expression of transgenes in the mammary tissue include promoters which naturally drive the expression of mammary-specific. For example, the αS1-casein promoters, αS2-casein promoters, β-casein promoters, κ-casein promoters, β-lactoglobulin promoters, whey acidic protein promoters, and α-lactalbumin promoters can be used. If desired, the promoter can be operably linked to one or more enhancer elements such that the enhancer element(s) increases transcription of the gene encoding the heterologous gene product.

The following references describe genes and expression control regions useful in the construction of transgenes in a variety of livestock: Groenen et al. (*Livestock Production Science* 38:61, 1994); Wilmut et al. (*Experientia* 47:905, 1991); Pursel et al. (*J. Animal Sci.* 71(suppl. 3):10, 1993); Clark et al. (U.S. Patent 5,322,775); and Bleck et al. (PCT Application No. PCT/US92/06549). Expression constructs and genes used in livestock other than goats can, if required, be adapted for use in goats. Hurwitz et al. (PCT Application No. PCT/US/06300) describes expression constructs

suitable for expression of a heterologous protein in the milk of a goat.

Preferably, the genetic construct (i.e., plasmid) also includes a transcription termination region. Useful termination regions include a polyadenylation signal and the 3'-end of the gene from which the promoter region of the genetic construct was derived. Other useful transcription termination regions include termination regions which are known to affect mRNA stability, such as those derived from the bovine growth hormone gene, globin genes, the SV40 early region or milk protein genes.

Optionally, the linear or circular genetic construct includes an intron which can increase the level of expression of the heterologous gene. Generally, the intron should be placed between the transcription initiation site and the translational start codon; 3' of the translational stop codon; or within the coding region of the gene encoding the heterologous protein. The intron should include a 5' splice site (i.e., a donor site), a 3' splice site (i.e., an acceptor site), and preferably includes at least 100 nucleotides between the two sites. Particularly useful introns are those which are naturally found in genes of ruminants (e.g., genes encoding caseins).

Heterologous Gene Products

Practically any heterologous protein can be produced in a transgenic dwarf goat. Particularly useful heterologous proteins include those which are of therapeutic value to humans or animals (e.g., htPA, hGH, and IL-6). Other particularly useful proteins include those which increase the nutritional value of the milk (e.g., β-casein and lactoferrin). Many genes encoding these and other useful proteins have been identified and cloned, allowing them to be readily subcloned for use in the production of transgenic dwarf goats.

Other particularly useful heterologous proteins include those which are valuable in food science. Among the useful proteins are those which possess an enzymatic activity directed toward a component of milk; such enzymes can be used to alter the lipid, protein, or carbohydrate content of the milk. For example, β-galactosidase can be produced with the invention to produce milk with a reduced lactose level. Genes encoding β-galactosidase can be derived from any of a number

of organisms, including *Aspergillus niger*, (Kumar et al., 1992, *Bio/technology* 10:82); *Homo sapiens* (Oshima et al., 1988, *Biochem. Biophys. Res. Comm.* 157:238); *Kluyveromyces lactis* (U.S. Pat. No. 5,047,340; Sreekrishna and Dickson, 1985, *Proc. Natl. Acad. Sci.* 82:7909; and Poch et al., 1992, *Gene* 118:55); *Lactobacillus bulgaricus* (Schmidt et al., 1989, *J. Bacteriology* 171:625).

Useful proteins include: cytokines, aspartic proteases, lysozyme, stearyl-CoA desaturase, lipases, galactosyltransferase, blood clotting proteins, protein C, α1-antitrypsin, urokinase plasminogen activator, human serum albumin, cystic fibrosis transmembrane conductance regulator, gamma-interferon, human CD4, growth factors, peptide hormones, oncoproteins, tumor suppressor proteins, milk proteins, hormone receptors, translation factors, and transcription factors.

If desired, the gene encoding the heterologous protein can be mutated. Particularly useful mutations include mutations in the 5'- or 3'- untranslated regions of the gene, because such mutations may improve expression of the gene encoding the heterologous protein. Other useful mutations or deletions are those which increase secretion of the protein from the cell or inhibit retention of the protein inside the cell. For example, sequences encoding endoplasmic reticulum retention signals or other sorting inhibitory signals are preferably deleted from the genetic construct or mutated to be non-functional. In addition, truncated versions of naturally-occurring proteins can be used in the invention, provided that the truncated protein possesses a useful biological activity.

Each heterologous protein produced according to the invention should be bonded to a signal peptide if the protein is to be secreted from the mammary epithelial cell. The signal peptide can be a naturally-occurring component of the heterologous protein (e.g., the signal peptide of human placental β-galactosidase). Where the heterologous protein is not naturally a secreted protein, if secretion is desired, the genetic construct should be assembled such that a signal peptide is bonded to the heterologous protein so that the signal peptide directs secretion of the protein from the

cell. Useful signal peptides can be derived from genes such as casein genes, the gene for human alkaline phosphatase, or the gene for melittin.

Tethered Enzymes

Where the genetic construct encodes an enzyme to be tethered to the epithelial cell membrane, the genetic construct can include a sequence encoding a membrane-associated polypeptide. For example, the genetic construct can include a sequence derived from β-1,4-galactosyltransferase (see, e.g., Masri et al., 1988, *Biochem. Biophys. Res. Comm.* 175:657-663), lactase-phlorizin hydrolase (see, e.g., Mantei et al., 1988, *EMBO J.* 7:2705-2713), the thy-1 protein (see, e.g., Brown et al., 1989, *Science*, 245:1499-1501), or the sodium/glucose transporter (see e.g., Kong et al., 1993, *FEBS Letters* 333:1-4).

Tethering an enzyme to the cell membrane allows the enzyme to be active on a component of milk, while inhibiting secretion of the enzyme into milk obtained from the mammal. Tethering the enzyme to the cell membrane can decrease the amount of heterologous enzyme that must be synthesized for modification of the milk components. While secreted enzymes remain in contact with milk in the bovine duct for approximately 12 hours before they are removed with the milk, the time span for tethered enzymes is limited only by the natural turnover of the epithelial cell membrane. Because the enzyme remains in the duct where it can act on milk produced over a long period of time, the cell may not need to synthesize as much of the heterologous enzyme as would be required if the heterologous enzyme were secreted. In addition, while milk obtained from such mammals can be modified as desired (e.g., to have a reduced lactose content), tethering the enzyme to the cell membrane decreases the concern that the modified milk contains an undesired heterologous enzyme.

Antisense Oligonucleotides

Where the genetic construct encodes an antisense oligonucleotide for inhibiting gene expression, the construct is engineered to produce an oligonucleotide which hybridizes to DNA or mRNA of the gene. Generally, this aspect of the

invention can be practiced using art-known antisense strategies. The DNA sequences of a number of mammary epithelial cell genes are known. Appropriate targets include, without limitation, genes encoding a β-lactoglobulin (see e.g., Ivanov et al., 1988; *J. Biol. Chem.* 369:425-429, and Silva et al., 1990, *Nucleic Acid Res.* 18:3015), an acetyl CoA carboxylase, a galactosyltransferase (Shaper et al., 1986, *Proc. Natl. Acad. Sci.* 83: 1573-1577; Masibay et al., 1989, *Proc. Natl. Acad. Sci.* 86: 5733-5737; Masri et al., 1988, *Biochem. Biophys. Res. Comm.* 175:657-663), and an α-lactalbumin (Vilotte et al., 1987, *Biochimie* 69:609-620). Thus, those skilled in molecular biology will be able to design a genetic construct encoding an antisense oligonucleotide of a suitable sequence and length. Preferably, the antisense oligonucleotide is between 5 and 1,000 nucleotides in length (more preferably 10 to 500 nucleotides).

Ribozymes

The gene transfer method of the invention can also be used to deliver genetic constructs encoding ribozymes to the mammary epithelial cells. Conventional strategies can be used in the design of genetic constructs encoding ribozymes. Appropriate target nucleic acids include, without limitation, those which encode β-lactoglobulin, acetyl CoA carboxylase, galactosyltransferase, and α-lactalbumin.

Genetic Knockouts

Using embryonic stem cell technology, one can knock out a selected gene.

Isolation and Characterization of Genetic Constructs

The genetic constructs useful in the invention can be prepared using conventional techniques for DNA isolation. If desired, DNA quality can be assessed with standard methods, such as measuring optical density or analyzing the DNA by electrophoresis. Preferably, the DNA is endotoxin free, and suitable methods include those which have been approved for purifying DNA for use in humans (e.g., the use of a Qiagen DNA extraction kit followed by the use of an endotoxin elimination kit). If desired, the genetic constructs can be further characterized by sequencing the DNA molecules, particularly at junctions formed by the ligation of two DNA molecules.

The creation of partial restriction maps from the genetic constructs can provide information regarding the orientation of the gene encoding the heterologous protein relative to the other components of the construct.

What is claimed is

Claims

1. A method comprising:

(a) introducing a transgene into a zygote of a dwarf goat,

(b) transplanting said zygote into a pseudopregnant non-dwarf goat, and

(c) allowing said zygote to develop to term.

2. A method comprising:

(a) introducing a transgene into an embryo of a dwarf goat,

(b) transplanting said embryo into a pseudopregnant non-dwarf goat, and

(c) allowing said embryo to develop to term.

3. A method comprising:

(a) introducing a transgene into an zygote of a dwarf goat,

(b) transplanting said zygote into a pseudopregnant dwarf goat, and

(c) allowing said zygote to develop to term.

4. A method comprising:

(a) introducing a transgene into an embryo of a dwarf goat,

(b) transplanting said embryo into a pseudopregnant dwarf goat, and

(c) allowing said embryo to develop to term.

5. The method of claim 1 or 2 further comprising:

(d) breeding said offspring to produce a transgenic dwarf goat.

6. The method of claim 2 wherein said introducing of said transgene into said embryo is by introducing an embryonic stem cell containing said transgene into said embryo.

7. The method of claim 2 wherein said introducing of said transgene into said embryo is by infecting said embryo with a retrovirus containing said transgene

8. The method of claim 1 wherein said introducing of said transgene into said zygote is by injecting the pronucleus of said zygote with said transgene.

9. The method of claim 1 wherein at least 4 said zygotes are transplanted into said pseudopregnant non-dwarf goat.

10. The method of claim 2 wherein at least 4 said embryos are transplanted into said pseudopregnant non-dwarf goat.

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/IB 96/01434

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A01K 67/027, A61D 19/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A01K, C12N, A61D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0350052 (DAIICHI PHARMACEUTICAL, LTD.), 10 January 1990 (10.01.90), page 4, line 14 - line 15, claim 5	1-10
X	EP, A2, 0247494 (THE GENERAL HOSPITAL CORPORATION), 2 December 1987 (02.12.87), claim 3	1-10
X	WO, A1, 9010699 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK), 20 Sept 1990 (20.09.90), claim 57	1-10
A	WO, A1, 8901972 (THE EDISON ANIMAL BIOTECHNOLOGY CENTER), 9 March 1989 (09.03.89), claim 1	1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 50, CAB Abstracts, Dialog accession no. 00021230, CAB accession no. 730104319, Sugie, T. et al: "The survival and development of fertilised ova transferred reciprocally between Saanen and Japanese native goats", Bulletin of the National Institute of Animal Industry, Japan (No. 23): 7-19 -- --------	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/02/97

International application No.
PCT/IB 96/01434

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		JP-A-	2131583	21/05/90
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		ES-T-	2090006	16/10/96
		JP-T-	1502716	21/09/89
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WO-A1- 9010699	20/09/90	AU-B-	653915	20/10/94
		AU-A-	5337890	09/10/90
		CA-A-	2048993	11/09/90
		EP-A-	0462215	27/12/91
		JP-T-	4504200	30/07/92
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		US-A-	5175385	29/12/92